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## VOLUME 66

IN HONOR OF

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# CHRONIC SELENIUM POISONING OF RATS AS INFLUENCED BY DIETARY PROTEIN

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(Received for publication October 16, 1939)

TWO FIGURES

In the course of preliminary experiments concerned with the nutritional aspects of selenium poisoning in rats, Lewis and Gortner ('37) observed that those animals receiving casein at a level of 30% in the diet were much less susceptible to the effects of added sodium selenite than were others receiving only 6% of dietary casein. This protective action of the high protein diet was manifested in the growth, longevity, and general appearance of the animals. About the same time Moxon ('37) reported that high protein diets afforded more protection against selenium than did diets low in protein. When rats were fed diets containing 10, 20, and 55% of casein together with 37.5 p.p.m. of selenium as sodium selenite, those receiving the most protein grew more than twice as fast as others on the low protein diet. Recently Smith ('39), in a detailed study of the influence of dietary constituents on chronic selenium intoxication in rats, observed again the protective action of high levels of protein. Selenium was present in these diets as the form occurring naturally in seleniferous flour.

The present experiments were undertaken in order to determine whether the earlier observed protection against ingested selenium by high levels of casein could be obtained with other proteins. Accordingly, diets containing such proteins as lactalbumin, gelatin, and edestin were studied in comparison with one high in casein and another low in protein content.

## EXPERIMENTAL

The compositions of the experimental diets are shown in table 1. The low protein diet used was the sulfur-deficient ration of White ('36) to which cystine was added in amounts sufficient to increase the sulfur content so as to be equal to that of the 30% casein diet. The various high protein diets were all derived from this basal diet; sufficient other protein was added to the 6% of casein already present to make a

TABLE 1

*Composition of experimental diets**

*The values indicate parts by weight and are approximate percentage units. In addition to these diets each rat received 3–4 drops of cod liver oil and one tablet (390 mg.) of pressed brewers' yeast daily.*

| DIET | $LP_{se}$ | $C_{se}$ | $G_{se}$ | $E_{se}$ | $L_{se}$ |
|---|---|---|---|---|---|
| Casein | 6 | 30 | 6 | 6 | 6 |
| Gelatin | 0 | 0 | 24 | 0 | 0 |
| Edestin | 0 | 0 | 0 | 24 | 0 |
| Lactalbumin | 0 | 0 | 0 | 0 | 24 |
| O–M salt mixture cont'g 875 p.p.m. Se | 4 | 4 | 4 | 4 | 4 |
| Sucrose | 15 | 15 | 15 | 15 | 15 |
| Cornstarch | 50 | 26 | 26 | 26 | 26 |
| Lard | 25 | 25 | 25 | 25 | 25 |
| Cystine | 0.68 | 0 | 0.68 | 0 | 0 |

* The proteins used were the following commercial products:
Casein—Labco vitamin-free casein
Gelatin—Eimer and Amend, U.S.P. granular
Edestin—Eimer and Amend, pure
Lactalbumin—Labco lactalbumin, No. 7HA

final concentration of 30% protein, this being substituted for an equivalent weight of starch. Selenium, as c.p. sodium selenite, was incorporated in the Osborne-Mendel salt mixture in such a concentration as to give a selenium level of 35 p.p.m. in all of the selenium-containing diets when fed. The selenium-free control diets were identical with those in the above table except that no selenium was incorporated in the salt-mixture. In figure 1 they are designated by the same letters as the seleniferous diets except that the subscript 'se' is omitted.

Young albino rats of inbred stock obtained from the Germantown, Pennsylvania colony were used throughout these experiments. At the age of 25 to 30 days they were transferred to individual raised-bottom wire cages and fed diet C for 4 to 7 days in order to accustom them to an artificial diet. When they were changed to control or seleniferous diets their weights ranged from 50 to 70 gm. The daily food intakes were recorded, and each rat was weighed twice weekly. The experiments were continued for periods ranging from 6 to 8 weeks,

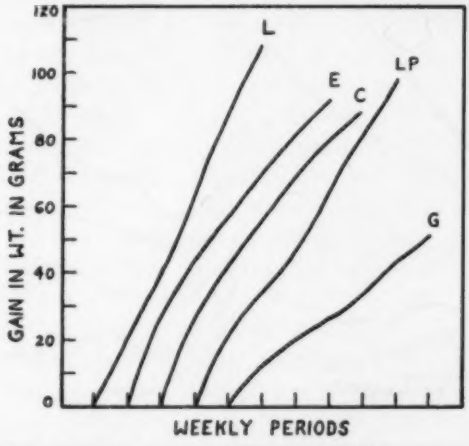


Fig. 1 Average growth curves for male control rats.
Each curve represents 2 or 3 animals. The letters with each curve designate the diet received.

The results of feeding the selenium-free control diets are shown in figure 1. It is evident that good growth is obtained with the low-protein diet as well as with those high in casein, lactalbumin and edestin. The rats receiving the high-gelatin diet, however, grew only about one-half as rapidly as the others, and their fur tended to become yellow and greasy in contrast to the animals on the other diets.

Figure 2 depicts the average growth curves of male and female rats on the 35 p.p.m. selenium diets. The same general

trends were observed for the two sexes with the exception that growth was not as pronounced in the females. Although only from 7 to 14 rats were placed on each of these diets, numbers admittedly not large, it is felt that the data are of such a consistent nature as to merit their being reported at this time. It should be emphasized that the diets high in lactalbumin, gelatin, and edestin all contained a basal level of

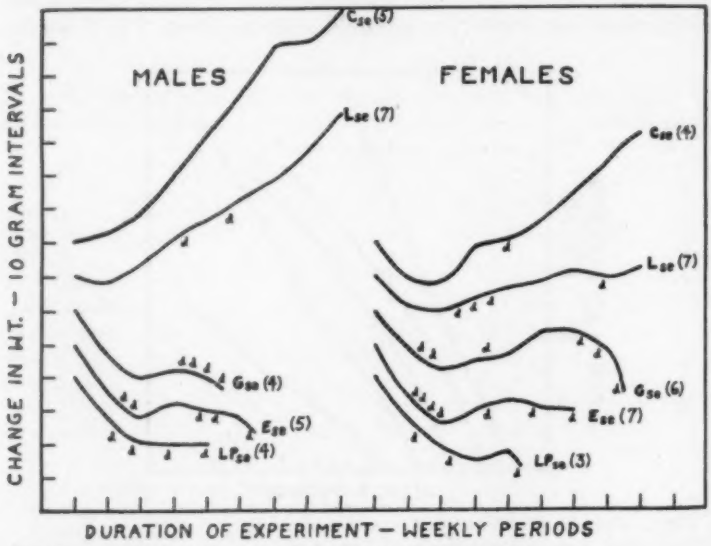


Fig. 2 Average growth curves for rats on seleniferous diets.

Numbers in parentheses represent number of animals on the diet. A small "d" indicates the death of an animal. The letters with each curve designate the diet received.

6% casein, so that the effects of these proteins on growth must be considered as supplementary effects.

It is seen that casein facilitated fair growth, and at the end of the 8-week experimental period all of the males and all but one of the females appeared healthy and were continuing to gain in weight. Examination of the internal organs at the end of this time showed gross pathological changes to a greater or

lesser extent in about 60% of the animals, the females being more severely affected than the males. The observed pathological changes included shrivelled, mottled, and granular appearance of the liver, hypertrophy of the spleen, and hypertrophy of the heart. Histological examinations of the livers showed some congestion. The testes were large and firm, and histological observations showed apparently normal spermatogenesis.

Lactalbumin also appeared to offset selenium intoxication to some extent, but the results were not so consistent as those obtained with casein. Of the seven males on the lactalbumin diet, four grew at a rate comparable to the rats receiving the high level of casein, while the others did poorly, two succumbing within 5 weeks. With the females, three showed fair growth during the experimental period while the remaining four expired before the end of this period. Accordingly, eight of the fourteen rats on this diet were alive and growing at the end of 8 weeks. The gross pathology of the internal organs was much the same for this group of rats as it was with those receiving the diet $C_{se}$.

With the other diets, however, the results were quite different. The edestin and gelatin diets failed to promote growth and offset the toxic manifestations of the selenium and were in nearly all respects comparable to the low-protein diet. In no instance did the survival period extend beyond 53 days, most of the deaths occurring between 9 and 32 days. Except for those animals which succumbed within 2 weeks after being placed on selenium-containing rations, gross pathology of the organs was more evident in these groups than in the groups receiving diets $C_{se}$ and $L_{se}$. Atrophy and cirrhosis of the liver were observed in 75% of the animals, and a similar percentage showed varying amounts of edemal fluid or blood in the pleural cavity, peritoneal cavity, or intestine. The blood was invariably thin and watery. The testes were flabby and undeveloped. Other organs such as the heart and spleen were usually very small but occasionally were decidedly hypertrophied.

Since gelatin is known to be practically devoid of tryptophane and sulfur, further experiments were carried out with fourteen rats in which 0.1% tryptophane and 0.84% methionine were included in a high-gelatin diet, the latter amino acid being substituted for the cystine in diet $G_{ss}$. However, the resulting failure of growth and early mortality were just as pronounced in this group as in the one that received the high gelatin plus cystine diet ($G_{ss}$).

The average daily intake of food varied greatly according to the diet. The animals on the non-seleniferous control diets

TABLE 2

*Average food and selenium intakes of male rats on control and on selenium-containing diets.*

| DIET | NUMBER OF RATS | AVERAGE DAILY FOOD INTAKE | | AVERAGE DAILY INTAKE OF Se | |
|---|---|---|---|---|---|
| | | Range | Average | Range | Average |
| | | *gm.* | *gm.* | *mg.* | *mg.* |
| LP | 2 | 7.3–9.5 | 8.4 | | |
| C | 2 | 7.2–7.5 | 7.4 | | |
| L | 2 | 6.2–7.6 | 6.9 | | |
| G | 3 | 6.6–7.2 | 6.9 | | |
| E | 2 | 7.1 | 7.1 | | |
| $LP_{ss}$ | 4 | 1.6–2.5 | 2.1 | 0.056–0.088 | 0.074 |
| $C_{ss}$ | 5 | 3.8–5.8 | 4.7 | 0.133–0.203 | 0.164 |
| $L_{ss}$ | 7 | 2.0–5.8 | 3.9 | 0.070–0.203 | 0.136 |
| $G_{ss}$ | 4 | 2.1–3.0 | 2.6 | 0.074–0.105 | 0.091 |
| $E_{ss}$ | 5 | 1.3–2.5 | 2.1 | 0.046–0.088 | 0.074 |

ingested, on the average, 6.5 to 7.5 gm. of food per day. When selenium was present in the diets, smaller amounts were consumed, as shown in table 2. It is evident from these data that, as has been pointed out previously by Franke and Potter ('35) and Franke ('35), growth is directly proportional to the food intake despite the increased consumption of selenium accompanying the higher levels of food intake.

## DISCUSSION

The data clearly indicate that the ability of casein, when fed at a high level, to counteract the symptoms of chronic selenium poisoning does not apply to all other proteins. When different proteins were used extreme differences in toxicity

were observed with seleniferous diets containing the same proportions of carbohydrate, fat, and protein. No satisfactory explanation of these results can be offered here. It seems doubtful that an explanation can be made on the basis of a deficiency in some amino acid, since edestin was selected because it is usually considered to be a rather 'complete' protein, i.e., one containing adequate amounts of the amino acids essential for normal growth. Perhaps the explanation of these discrepancies lies in the ease with which the various proteins are digested and absorbed from the intestinal tract; no studies were made in this respect.

Although the various high-protein diets had similar selenium concentrations and caloric values, the daily food intakes of the animals varied markedly. Animals on the casein and lactalbumin rations ingested about twice as much selenium per day as did those on other diets, yet this additional intake of the toxicant was more than counteracted by the increased protein consumption. Of these two proteins, lactalbumin was less consistent in offsetting the selenium toxicity.

Smith ('39) has observed that a high-casein diet containing 10 p.p.m. of selenium caused no impairment of growth, and affected but slightly the ability to reproduce and the macroscopic appearance of the liver and other organs. In his experiments the selenium was bound organically in seleniferous flour, whereas in the present study sodium selenite was employed. Figure 2 indicates that when inorganic selenium is present in the diet to the extent of 35 p.p.m., the growth is somewhat curtailed even when large amounts of casein are fed. Autopsies performed at the conclusion of the experimental periods showed striking pathological changes in the livers of many of these animals, including many of those which appeared normal and were rapidly gaining in weight at the time. The observations (Smith, '39) that female rats are more susceptible than males to liver injury and that dietary casein tends to prevent atrophy of the reproductive organs have been confirmed in the present study.

It would be interesting to know what the reaction of rats would be to seleniferous diets high in zein, hordein, oryzenin,

gliadin and glutenin, since these proteins occur naturally in the toxic grains from the seleniferous districts in the Great Plains region. An extension of this work using certain of these and other proteins is contemplated.

## SUMMARY

Earlier observations on young white rats dealing with the protective action of high levels of casein against chronic selenium poisoning have been confirmed. Good growth results when as much as 35 p.p.m. of selenium, as sodium selenite, is incorporated in a diet containing 30% of casein. Only one rat, a female, died during the 8-week experimental period.

The ability of lactalbumin, gelatin, and edestin to render similar protective action to rats against ingested selenium (35 p.p.m.) has been tested. These proteins were all superimposed on a basal level of 6% casein in amounts sufficient to give 30% of protein in the diets.

Lactalbumin tends, as does casein, to counteract the toxic effects of dietary selenium, but it does not give as consistently good results as does casein.

Edestin and gelatin, when fed at high levels in seleniferous diets, exert no detoxifying action and are in all respects comparable to a diet containing only 6% of casein. Rats on these diets failed to grow and died in most cases within 32 days. None of these animals lived beyond 53 days.

Pathological changes have been described.

## LITERATURE CITED

FRANKE, K. W. 1935 A new toxicant occurring naturally in certain samples of plant foodstuffs. X. The effect of feeding toxic and control foodstuffs alternately. J. Nutrition, vol. 10, p. 233.

FRANKE, K. W., AND V. R. POTTER 1935 A new toxicant occurring naturally in certain samples of plant foodstuffs. IX. Toxic effects of orally ingested selenium. J. Nutrition, vol. 10, p. 213.

LEWIS, H. B., AND R. A. GORTNER, JR. 1937 Unpublished data.

MOXON, A. L. 1937 Alkali disease or selenium poisoning. So. Dak. Agr. Exp. Sta. Bul. 311, p. 50.

SMITH, M. I. 1939 The influence of diet on the chronic toxicity of selenium. Pub. Health Rep., vol. 54, p. 1441.

WHITE, A. 1936 The production of a deficiency involving cystine and methionine by the administration of cholic acid. J. Biol. Chem., vol. 112, p. 503.

# A COMPARISON OF THE UTILIZATION BY GUINEA PIGS OF EQUIVALENT AMOUNTS OF ASCORBIC ACID (VITAMIN C) IN LEMON JUICE AND IN THE CRYSTALLINE FORM [1]

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(Received for publication October 10, 1939)

The identification of vitamin C as ascorbic acid and the synthesis of this substance have led to the development of titration methods for the quantitative estimation of vitamin C in biological tissues and fluids. It also has made possible the study of the comparative utilization by the body of ascorbic acid as it occurs in the natural state in foods and of the crystalline form of the vitamin.

Jacobsen ('35) reported a lower concentration of ascorbic acid in the adrenals of guinea pigs receiving 20 mg. daily of crystalline ascorbic acid, than in the same organs of animals receiving cabbage ad libitum daily; the cabbage was stated "to contain something like 20 mg. of ascorbic acid per day."

Fox and Levy ('36) found that dehydroascorbic acid was effective as an antiscorbutic but that it was not as easily stored as the parent substance. They also reported that the vitamin C present in the green leaves of lucerne was comparable to orange juice in its availability to the guinea pig. However, their data showed that four animals fed for 2 months on a basal diet plus 5 ml. of orange juice equivalent to 2.5–3.0 mg. of ascorbic acid per day had an average store of 0.50 mg. of ascorbic acid per gram of adrenal tissue as compared with five animals fed for 3 months on a basal diet plus lucerne leaves

[1] Published as scientific paper no. 421, Agricultural Experiment Station, State College of Washington.

equivalent to 3.2 mg. of ascorbic acid per day, which had an average store of only 0.32 mg. per gram of adrenals.

Giroud, Leblond and Ratsimamanga ('37) reported on the stores of ascorbic acid in the adrenal glands of guinea pigs receiving their supply of the vitamin from beet leaves, alfalfa, and cabbage leaves and from pure ascorbic acid. However, the ascorbic acid was given in solution in orange juice and the animals received a different basal diet from those receiving vegetable supplements; comparable levels of ascorbic acid were not fed in each group. The ascorbic acid of beet leaves was reported to be less well utilized than that of alfalfa and cabbage leaves; 6.6 mg. of ascorbic acid daily as beet leaves gave a storage of 0.448 mg. per gram of adrenal, while 5 mg. of ascorbic acid as alfalfa produced an adrenal store of 0.695 mg. per gram. Alfalfa leaves supplying 40 mg. of ascorbic acid daily gave an adrenal store of 1.069 mg. per gram while a 50 mg. level of pure ascorbic acid dissolved in orange juice produced only 1.055 mg. per gram.

Hawley, Daggs and Stephens ('37) reported that there was better retention of ascorbic acid in the tissues of guinea pigs when the vitamin was ingested in the natural form as cabbage, alfalfa hay, and orange juice than when taken in the form of crystalline vitamin C.

This investigation has been undertaken with the hope of throwing further light on the question of utilization of ascorbic acid from different sources, by making direct comparison of two forms of the vitamin fed at the same level to comparable animals.

## EXPERIMENTAL

Two series of guinea pigs were used, and in each case, ascorbic acid in crystalline form [2] was compared with an equivalent amount of ascorbic acid in the form of lemon juice.

[2] Acknowledgment is made to Merck and Company for a generous supply of ascorbic acid.

Comparisons have been made on the basis of weight gains, scurvy scores, and the ascorbic acid content of the blood and of the adrenal glands.

*Series I.* The guinea pigs were 6 to 8 weeks of age and weighed approximately 300 gm. All were shown to be healthy and actively growing at the beginning of the experiment. The vitamin C-free basal diet was the same as that described by Todhunter ('36). The animals, eighteen in number, were caged separately, and weighed every other day. They remained on the basal diet for 13 days and then were given the supplementary feedings for 20 days, and were killed on the twenty-first day.

One group of nine animals was given 1 ml. of lemon juice daily, fed directly into the mouth of the animal from a graduated glass syringe. The ascorbic acid content of the lemon juice was determined daily by titration with 2,6 dichlorophenolindophenol; a solution of pure ascorbic acid was then prepared of such concentration that 1 ml. of this solution was equivalent in ascorbic acid content to 1 ml. of lemon juice for that day. A second group of nine animals received daily 1 ml. of this ascorbic acid solution. The average daily intake for the group was 0.47 mg. of ascorbic acid in 1 ml. of lemon juice, and the same amount of the pure substance in aqueous solution.

Just before death, 1 ml. of blood was removed from the heart of the anesthetized animal. Preliminary studies showed that there was no change in level of blood ascorbic acid of animals kept under ether anesthetic for 7 minutes. The determination of ascorbic acid content of the blood was by the micro method of Farmer and Abt ('36).

The degree of scurvy was rated at death according to the scoring method of Sherman and co-workers ('22).

The adrenals were removed immediately, washed in saline solution, dried on filter paper, weighed, and ground with acid-washed sand and a 3% solution of metaphosphoric acid. After centrifuging, decanting and washing twice, the combined extracts from the adrenals were titrated with 2,6 dichlorophenol-

indophenol solution which had been standardized against pure ascorbic acid.

The results are summarized in table 1. All of the animals were of the same weight at the beginning, and the average

TABLE 1

*Data of animals receiving daily 1 ml. lemon juice or an equivalent solution of ascorbic acid*

| GUINEA PIG NO. | BODY WEIGHT | | | SCURVY SCORE | ADRENAL WEIGHT | ASCORBIC ACID IN ADRENALS | | ASCORBIC ACID PER 100 ML. PLASMA |
|---|---|---|---|---|---|---|---|---|
| | Initial | 14-day period | Final | | | Total | Per gram | |
| | gm. | gm. | gm. | | gm. | mg. | mg. | mg. |
| Lemon juice 1 ml. daily | | | | | | | | |
| 640 ♀ | 309 | 354 | 378 | 1 | 0.2438 | 0.023 | 0.094 | 0.23 |
| 602 ♂ | 293 | 339 | 414 | 1 | 0.1904 | 0.018 | 0.095 | 0.18 |
| 597 ♂ | 297 | 375 | 424 | 1 | 0.3023 | 0.024 | 0.079 | 0.16 |
| 679 ♀ | 285 | 303 | 327 | 7 | 0.2369 | 0.018 | 0.077 | 0.16 |
| 668 ♂ | 305 | 362 | 390 | 5 | 0.2201 | 0.016 | 0.073 | 0.10 |
| 669 ♂ | 308 | 356 | 394 | 1 | 0.2801 | 0.018 | 0.065 | 0.10 |
| 728 ♀ | 285 | 331 | 395 | tr | 0.3179 | 0.036 | 0.113 | 0.1? |
| 745 ♀ | 290 | 313 | 331 | 7 | 0.1557 | 0.015 | 0.038 | 0.13 |
| 735 ♂ | 280 | 316 | 385 | 3 | 0.1891 | 0.021 | 0.112 | 0.14 |
| Average | 295 | 339 | 382 | | 0.2374 | 0.021 | 0.090 | 0.15 |
| S.D. | | | | | ±0.0549 | ±0.006 | ±0.017 | ±0.04 |
| Ascorbic acid solution 1 ml. daily | | | | | | | | |
| 607 ♀ | 291 | 279 | 294 | 4 | 0.2622 | 0.023 | 0.088 | 0.14 |
| 639 ♂ | 307 | 358 | 452 | 2 | 0.2743 | 0.034 | 0.124 | 0.21 |
| 615 ♂ | 294 | 352 | 406 | 2 | 0.2410 | 0.023 | 0.095 | 0.11 |
| 677 ♂ | 312 | 326 | 287 | 8 | 0.2221 | 0.017 | 0.076 | 0.14 |
| 678 ♀ | 290 | 318 | 390 | 6 | 0.2112 | 0.038 | 0.180 | 0.20 |
| 683 ♀ | 282 | 295 | 337 | 8 | 0.2772 | 0.038 | 0.139 | 0.19 |
| 747 ♂ | 285 | 313 | 352 | 6 | 0.1917 | 0.021 | 0.109 | 0.13 |
| 734 ♂ | 320 | 368 | 475 | 1 | 0.2689 | 0.038 | 0.141 | 0.07 |
| 730 ♂ | 287 | 274 | 362 | 6 | 0.1920 | 0.015 | 0.078 | 0.11 |
| Average | 296 | 320 | 372 | | 0.2378 | 0.027 | 0.114 | 0.14 |
| S.D. | | | | | ±0.0347 | ±0.010 | ±0.035 | ±0.05 |

weight for each group was approximately the same at the end of the period. No difference was noted in either the average adrenal weights, or in the total ascorbic acid content of the adrenals of the two groups, and the ascorbic acid content of the blood plasma was the same. There was a wide variation

in the scurvy scores of individual animals in each group, but there were more low scores in the lemon juice group.

*Series II.* Since no difference in the growth, or body stores of ascorbic acid was found in the animals receiving such a low level of the vitamin, it was decided to see whether differences could be observed at a higher level of intake of the vitamin. Two milliliters of lemon juice were believed to be the amount which a young animal could reasonably consume at one time without introducing a possible error due to lack of absorption or intestinal destruction through delayed absorption. Therefore, the second series of animals received daily a total of 4 ml. of lemon juice fed in two portions.

Twelve animals were used in this series and they were comparable in age and weight to those of the first series. During the preliminary period when the guinea pigs were attaining the required weight of 300 gm., all animals received the basal diet plus spinach and carrots ad libitum. These animals were not made scorbutic first as in series I but, in an attempt to equalize the vitamin C reserves at the beginning of the experiment, all were kept for 1 day on the basal diet only, and then for 3 days, each received in addition 1 ml. of lemon juice daily. The animals were then divided in two groups: the first group received, daily, 4 ml. of lemon juice, 2 ml. being fed in the morning and 2 ml. at noon; the second group received 2 ml. of ascorbic acid solution in the morning and 2 ml. at noon, and this solution was equivalent in ascorbic acid content to the lemon juice. The daily feedings of the supplement were continued for 30 days.

The average ascorbic acid content of the 4 ml. of lemon juice which was fed daily during the experimental period was 1.80 mg. and therefore, the average intake of ascorbic acid from either source averaged 1.80 mg. daily for 30 days. At the end of the experimental period, the animals were killed, scored for scurvy and analyses of blood and adrenals were made as described for series I. The data for this group of animals are summarized in table 2. The animals receiving lemon juice showed no signs of scurvy, but four of the animals

receiving crystalline ascorbic acid had some macroscopic traces of hemorrhage in the joints or muscles. The ascorbic acid content of the blood plasma of both groups was the same and was higher than that of the animals in series I. The adrenal glands were smaller in the two groups of series II and this is in agreement with the findings of other investigators

TABLE 2

*Data of animals receiving daily 4 ml. lemon juice or an equivalent solution of ascorbic acid*

| GUINEA PIG NO. | BODY WEIGHT | | SCURVY SCORE | ADRENAL WEIGHT | ASCORBIC ACID IN ADRENALS | | ASCORBIC ACID PER 100 ML. PLASMA |
|---|---|---|---|---|---|---|---|
| | Initial | Final | | | Total | Per gram | |
| | *gm.* | *gm.* | | *gm.* | *mg.* | *mg.* | *mg.* |
| Lemon juice, 4 ml. daily | | | | | | | |
| 753 ♂ | 300 | 491 | 0 | 0.1344 | 0.049 | 0.367 | 0.29 |
| 761 ♂ | 306 | 519 | 0 | 0.3851 | 0.117 | 0.303 | 0.22 |
| 785 ♀ | 300 | 323 | 0 | 0.1671 | 0.022 | 0.130 | 0.21 |
| 773 ♂ | 298 | 440 | 0 | 0.2194 | 0.050 | 0.227 | 0.19 |
| 755 ♂ | 293 | 287 | 0 | 0.1672 | 0.021 | 0.124 | 0.26 |
| 784 ♀ | 312 | 407 | 0 | 0.2114 | 0.046 | 0.217 | 0.21 |
| Average | 301 | 411 | | 0.2141 | 0.051 | 0.228 | 0.23 |
| S.D. | | | | ±0.0894 | ±0.035 | ±0.095 | ±0.04 |
| Ascorbic acid solution, 4 ml. daily | | | | | | | |
| 757 ♀ | 295 | 281 | tr | 0.2155 | 0.016 | 0.076 | 0.23 |
| 763 ♀ | 300 | 310 | 0 | 0.2375 | 0.030 | 0.128 | 0.17 |
| 767 ♂ | 306 | 560 | 0 | 0.1397 | 0.048 | 0.343 | 0.25 |
| 752 ♂ | 296 | 368 | tr | 0.1763 | 0.043 | 0.246 | 0.22 |
| 783 ♀ | 294 | 420 | tr | 0.3230 | 0.037 | 0.115 | 0.19 |
| 759 ♂ | 292 | 392 | tr | 0.2594 | 0.070 | 0.268 | 0.24 |
| Average | 299 | 388 | | 0.2252 | 0.041 | 0.196 | 0.22 |
| S.D. | | | | ±0.0643 | ±0.018 | ±0.104 | ±0.03 |

(Quick, '33; Hou, '34) that the adrenal gland becomes enlarged in scurvy. The amount of ascorbic acid stored in the adrenal gland was greater in both groups of animals of series II. The adrenals of the animals receiving 4 ml. of lemon juice showed a higher average content of ascorbic acid than those of the animals fed the equivalent ascorbic acid solution, but the difference is probably too small to be considered significant.

At the levels of ascorbic acid intake used in this study the lemon juice and crystalline ascorbic acid appear to be equally well utilized as a source of vitamin C when judged by the blood level and storage of ascorbic acid in the adrenal glands. However, the animals receiving crystalline ascorbic acid showed more evidence of hemorrhages and therefore, had higher scurvy scores than those receiving lemon juice. Bentsáth and co-workers ('36) reported that vitamin P was a factor in the maintenance of normal capillary resistance but Zilva ('37) failed to confirm this finding. The existence of vitamin P has yet to be conclusively demonstrated but Elmby and Warburg ('37) have found that ascorbic acid alone fails to cure the hemorrhagic condition of human scurvy.

The data presented in this paper, though not conclusive, do indicate the possibility that lemon juice may contain another factor concerned in the prevention of the hemorrhages which are characteristic of scurvy.

## SUMMARY

Animals fed comparable amounts of ascorbic acid [3] in aqueous solution, and as lemon juice made similar gains in weight, but those receiving lemon juice had fewer hemorrhages when scored for scurvy.

The blood plasma levels were the same and there was no appreciable difference in the ascorbic acid content of the adrenals of each group.

The data indicate the possibility that lemon juice contains an additional factor which is concerned in the prevention of the hemorrhages characteristic of scurvy.

## LITERATURE CITED

BENTSÁTH, A., ST. RUSZNYAK AND A. SZENT-GYORGYI 1936 Vitamin nature of flavones. Nature, vol. 138, p. 798.

ELMBY, A., AND E. WARBURG 1937 The inadequacy of synthetic ascorbic acid as an antiscorbutic agent. Lancet, vol. 11, p. 1363.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. and Med., vol. 34, p. 146.

[3] See footnote on page 114.

FOX, F. W., AND L. F. LEVY 1936 Experiments confirming the antiscorbutic activity of dehydroascorbic acid and a study of its storage and that of ascorbic acid by the guinea pig at different levels of intake. Biochem. J., vol. 30, p. 211.

GIROUD, A., C. P. LEBLOND AND R. RATSIMAMANGA 1937 The vitamin C requirement of the guinea pig. Yale J. Biol. Med., vol. 9, p. 573.

HAWLEY, E. E., R. G. DAGGS AND D. J. STEPHENS 1937 The effect of the administration of acid and alkaline salts upon the ascorbic acid content of guinea pig tissues. J. Nutrition, vol. 14, p. 1.

HOU, H. C. 1934 The relationship of adrenal weights to scurvy in guinea pigs. Trans. 9th Congress Far East. Assoc. Trop. Med., vol. 2, p. 693.

JACOBSEN, E. 1935 On the storage of ascorbic acid in the organs of guinea pigs given crystalline ascorbic acid with a vitamin C-free diet. Skand. Arch. Physiol., vol. 72, p. 259.

QUICK, A. J. 1933 Hypertrophy of the adrenals in scurvy. Proc. Soc. Exp. Biol. and Med., vol. 30, p. 753.

SHERMAN, H. C., V. K. LAMER AND H. L. CAMPBELL 1922 The quantitative determination of the antiscorbutic vitamin (vitamin C). J. Am. Chem. Soc., vol. 44, p. 165.

TODHUNTER, E. N. 1936 Some factors influencing ascorbic acid (vitamin C) content of apples. Food Research, vol. 1, p. 435.

ZILVA, S. S. 1937 Vitamin P. Biochem. J., vol. 31, p. 915.

# A COMPARISON OF THE UTILIZATION BY COLLEGE WOMEN OF EQUIVALENT AMOUNTS OF ASCORBIC ACID (VITAMIN C) IN RED RASPBERRIES AND IN CRYSTALLINE FORM[1, 2]

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In the preceding paper it was shown that vitamin C in the form of lemon juice or crystalline ascorbic acid was equally well utilized by guinea pigs when fed at levels of 0.5 mg. and 2 mg. daily and judged by weight gains, the storage of ascorbic acid in the adrenal glands, and the blood level of ascorbic acid.

The investigation here reported was undertaken to determine whether there was any measurable difference in the utilization by college women of ascorbic acid as it occurs naturally in red raspberries and in the crystalline form.

Ascorbic acid is not excreted in the urine in any appreciable amounts unless the requirements of the organism are fully met. Similarly, the blood plasma content of ascorbic acid falls almost immediately if the intake is inadequate, if there is incomplete absorption, or if there is destruction of the vitamin in the intestinal tract or other parts of the body. The technic used in this investigation, while it does not throw further light on the problem of what happens to ascorbic acid within the

[1] Published as scientific paper no. 422, Agricultural Experiment Station, State College of Washington.

[2] This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

[3] The data of this study are taken from a thesis submitted by Alva Fatzer in partial fulfillment of the requirements of the degree of master of science.

system, nevertheless, does serve as a measure of the comparative utilization of the vitamin from different sources; a fall in ascorbic acid content of either of the body fluids studied would indicate an intake inadequate to meet the metabolic needs of the organism.

Hawley and co-workers ('36) found that approximately the same amount of vitamin C was excreted in the urine when 100 mg. of ascorbic acid was ingested as orange juice or as crystalline ascorbic acid.

Elmby and Warburg ('37) reported that a total intake of 6 gm. of ascorbic acid failed to cure the hemorrhagic condition present in three patients with scurvy, but the juice of ten lemons daily for 10 days was therapeutically effective. They have suggested that some unknown substance, a co-vitamin, is required for the absorption and retention of ascorbic acid, and that this substance may either be part of some foodstuff or may be produced in the intestine under normal conditions.

McGovern, Gannon and Wright ('39) have also reported lack of success of oral intake of ascorbic acid as a cure for scurvy in some subjects unless the dose was extremely high.

## EXPERIMENTAL

The urinary excretion and the blood plasma content of ascorbic acid were the criteria for studying the utilization of ascorbic acid.

### *Subjects*

Seven college women, graduate students and faculty, served as subjects and gave full cooperation throughout the entire study. The age, height and weight of the subjects were as follows: A, 23 years, 162.0 cm., 51.26 kg.; B, 29 years, 161.0 cm., 51.71 kg.; C, 36 years, 161.3 cm., 62.60 kg.; D, 28 years, 154.0 cm., 49.90 kg.; E, 31 years, 157.5 cm., 61.69 kg.; F, 36 years, 165.1 cm., 60.33 kg.; G, 37 years, 173.5 cm., 66.68 kg.

### *Experimental period*

The study was divided into four consecutive periods as follows:

*Preliminary:* Subjects ate their usual diets for 3 days.

*Period I:* Consisted of 3 days during which time the subjects remained on their usual diets plus the juice of five oranges each day. The orange juice was taken in two portions, at breakfast and at noon, and supplied approximately 200 mg. of ascorbic acid. At the end of this period the subjects were assumed to be saturated with vitamin C.

*Period II:* Subjects continued for 6 days on a basal diet supplying approximately 20 mg. ascorbic acid per day, supplemented with a daily portion of frozen red raspberries containing 40 mg. of ascorbic acid.

*Period III:* The usual diet was again resumed for 3 days with addition of orange juice as in period I, to bring the subject to a state of saturation.

*Period IV* was similar to period II except that the daily supplement was 40 mg. crystalline ascorbic acid.[4]

### *Basal diet*

The basal diet used in this study was that recommended by Hauck ('38) and consisted of: 60 gm. American cheese, 100 gm. canned carrots plus 10 gm. juice, 60 gm. cooked dried prunes plus 10 gm. juice, 100 gm. canned pears plus 50 gm. juice, 100 gm. canned whole beets plus 10 gm. juice, 100 gm. ground beef, 60 gm. evaporated milk. The following foods were used ad libitum: Ry-Krisp, whole wheat cereal, eggs, rice, butter, nuts, sugar, whole wheat and white flour. The water intake was approximately equalized for all subjects and consisted of one cup of coffee at breakfast and five cups of water throughout the day at regular intervals. The canned foods were purchased in one lot for the entire study and the same brand was used throughout. Samples of the fruits, vegetables, milk and meat were analyzed for vitamin C by titration with 2,6 dichlorophenolindophenol. Assuming that the reducing value determined by titration was due solely to

[4] Acknowledgment is made to Merck and Company for a generous supply of ascorbic acid.

vitamin C the portion of each food consumed daily contributed the following amounts of ascorbic acid: pears and juice 2.8 mg., beets and juice 11.1 mg., evaporated milk 0.7 mg., carrots and juice 1.6 mg., prunes 2.8 mg., beef 1.0 mg., giving a total intake of ascorbic acid from the basal diet of 20 mg. per day. The basal diet was estimated from tables of food composition to supply: 47.7 gm. protein, 0.863 gm. calcium, 0.983 gm. phosphorus, 9.78 mg. iron. Adequate calories were obtained from the ad libitum foods, and the liberal use of butter and whole grain cereals probably supplied sufficient vitamins A and B; the evaporated milk was irradiated.

All meals were prepared and eaten in the college diet kitchen.

### *Dietary supplements*

*Red raspberries.* The red raspberries were of the Antwerp variety grown at the Western Washington Experiment Station,[5] frozen-packed immediately after harvesting and stored at 0° F. The berries were packed in no. 2 enamel-lined cans; as required, cans were opened and the contents weighed out so that each portion contained 40 mg. ascorbic acid. The berries were eaten at breakfast in the partially thawed state.

The ascorbic acid content of the berries was determined by titration [6] with 2,6 dichlorophenolindophenol according to the method of McHenry and Graham ('35).

*Ascorbic acid.* In period IV the supplement of 40 mg. ascorbic acid was taken in the form of the crystalline substance dissolved in a small amount of distilled water. The ascorbic acid solution was taken at breakfast time.

### *Urinary collection and analysis*

Urine was collected at the end of the first 6 hours of the morning excretion and for the remaining 18 hours. Collection

[5] Acknowledgment is made to Dr. C. D. Schwartze for the supply of raspberries used in this study and to H. C. Diehl, Frozen Pack Laboratory, U. S. Bureau of Chemistry and Soils at Seattle, Washington, for freezing the fruit.

[6] Acknowledgment is made to Ruth C. Robbins for determination of the ascorbic acid content of the red raspberries.

was made in 1 quart jars containing the following amounts of a preservative recommended by Sendroy ('37): 75 ml. of 5 N. sulphuric acid, 0.75 ml. 8-hydroxyquinoline (1.45 gm. in 100 ml. of ethyl alcohol) and 5 ml. toluene.

Recovery tests on samples of urine with this preservative showed that it permitted negligible destruction of ascorbic acid during the 24-hour period. The ascorbic acid content of the urine was determined by titration with 2,6 dichlorophenol-indophenol which had been standardized against a solution of pure ascorbic acid. The dye solution was of such strength that 2 to 5 ml. were required for the titration of aliquots of 10 or 20 ml. of urine, depending on the concentration of ascorbic acid in the urine. Titrations were completed in less than 2 minutes and the end point was taken as a faint pink color lasting 10 seconds.

### *Blood analysis*

Blood ascorbic acid was determined by the micro-method of Farmer and Abt ('36). Blood samples were taken from a finger prick while the subject was in the post-absorptive state and analyses were made at the end of the preliminary period and the beginning and end of period II and IV. Subjects A and B were tested for kidney function by injection of phenol-sulphonaphthalein and were found to be normal.

## RESULTS AND DISCUSSION

In the preliminary period on their usual diets the subjects showed a variable excretion of ascorbic acid, the lowest level being 9 mg. for subject B as seen from table 1. All subjects immediately gave a marked rise in excretion the first day that oranges were added which would indicate that even those subjects with a previously low excretion of vitamin C were yet in a good state of vitamin C nutrition. The blood plasma levels confirmed this (table 2), subject A showing 0.91 mg. per 100 ml. of plasma and all the others above 1 mg. per 100 ml.; a level of 0.8 mg. or higher is generally accepted as indicating

a satisfactory state of vitamin C nutrition. Therefore the level of urinary excretion of subjects on their usual diets cannot alone serve as an indicator of vitamin C 'saturation.' In both periods II and III when orange juice was added to the basal diet the subjects were 'saturated' with vitamin C as judged by the increased urinary excretion and blood plasma content

TABLE 1

*Excretion of ascorbic acid by subjects on basal diet plus 40 mg. of crystalline ascorbic acid and an equivalent amount from red raspberries*

| DIETARY PERIOD | MILLIGRAMS OF ASCORBIC ACID EXCRETED PER 24 HOURS FOR SUBJECTS: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | $B_1$ | $B_2$ | C | D | E | F | G |
| Usual [1] | 70 | 9 | 21 | 28 | 98 | 63 | 28 | 103 |
| Usual + oranges [1] | 271 | 160 | 127 | 267 | 205 | 241 | 316 | 262 |
| Basal + raspberries | | | | | | | | |
| 1st day | 87 | 22 | 21 | 101 | 46 | 74 | 73 | 47 |
| 2nd day | 77 | 36 | 22 | 53 | 40 | 44 | 40 | 52 |
| 3rd day | 59 | 35 | 18 | 44 | 28 | 40 | 26 | 22 |
| 4th day | 45 | 10 | 5 | 39 | 40 | 14 | 18 | 20 |
| 5th day | 31 | 29 | 15 | 22 | 29 | 22 | 16 | 26 |
| 6th day | 32 | 13 | 8 | 18 | 22 | 25 | 13 | 16 |
| Usual + oranges [1] | 271 | 154 | 175 | 260 | 211 | 220 | 275 | 214 |
| Basal + ascorbic acid | | | | | | | | |
| 1st day | 58 | 43 | 29 | 83 | 68 | 45 | 72 | 27 |
| 2nd day | 40 | 17 | 25 | 44 | 43 | 34 | 47 | 16 |
| 3rd day | 34 | 18 | 16 | 24 | 24 | 20 | 26 | 22 |
| 4th day | 11 | 9 | 6 | 18 | 22 | 14 | 22 | 21 |
| 5th day | 28 | 10 | 10 | 16 | 15 | 17 | 19 | 14 |
| 6th day | 16 | 13 | 13 | 16 | 25 | 11 | 12 | 19 |

[1] Ascorbic acid value for the last day only of each of these periods is given.

TABLE 2

*Ascorbic acid content of blood plasma of subjects on the last day of each dietary period*

| DIETARY PERIOD | MILLIGRAMS OF ASCORBIC ACID PER 100 ML. BLOOD PLASMA OF SUBJECTS: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | $B_1$ | $B_2$ | C | D | E | F | G |
| Usual | 0.91 | 1.03 | 0.99 | 0.86 | 1.65 | 1.39 | 1.66 | 1.45 |
| Usual + orange juice | 1.78 | 1.86 | 1.83 | 1.89 | 1.68 | 1.69 | 1.94 | 1.69 |
| Basal + raspberries | 1.45 | 0.92 | 1.17 | 1.42 | 1.26 | 1.10 | 1.13 | 1.19 |
| Usual + oranges | 2.14 | 2.27 | 1.68 | 1.56 | 1.56 | 1.79 | 1.80 | 1.47 |
| Basal + ascorbic acid | 1.80 | 1.46 | 1.09 | 1.14 | 1.22 | 1.11 | 1.14 | 1.10 |

of ascorbic acid. All were, therefore, in a comparable state at the beginning of each of the two test periods of receiving the basal diet plus 40 mg. ascorbic acid and the equivalent amount of frozen packed red raspberries.

During each of these test periods, the urinary excretion fell for the first 3 or 4 days and then became more constant, though there was considerable fluctuation for each individual even on this uniform daily intake. This daily fluctuation makes it somewhat difficult to compare the excretion during the two periods. Sendroy and Schultz ('36) proposed a utilization index, I, for comparing the state of ascorbic acid nutrition of various individuals. This index takes into consideration the body weight and age of the subject. Sendroy reported the normal utilization index to vary only within average limits of $67.5 \pm 5.5$ and he arrived at this figure from a study of the output of subjects receiving 250 mg. ascorbic acid daily for 7 days. Since the two test periods for each subject in this investigation were exactly the same with regard to vitamin C intake, the only variable being the source of vitamin C, it was considered justifiable to use Sendroy's formula for comparing these data, even though the previous histories were different and the level of intake was lower than Sendroy had used. The smaller the difference between output and intake of vitamin C, the better the utilization and the smaller the resultant figure for the utilization index.

The utilization indexes are summarized in table 3 and show for all subjects, except A and C, a comparable value for the two test periods. Subjects A and C each had a lower index during the period when raspberries were the supplement, indicating that there was probably better utilization of the ascorbic acid of the berries by these two subjects.

For each subject, the raspberry supplement period preceded that when ascorbic acid was used and it was considered possible that there might be some cumulative effect of ascorbic acid intake in raising the blood level at the end of the experiment since A and B, the first two subjects on the study, showed a higher blood value (table 2) at the end of the ascorbic acid

period than at the end of the raspberry period. Subject B therefore repeated the experiment reversing the order of taking the supplements in the two test periods; this had no influence on the results as is shown in tables 1 and 2, where the repeat experiment for this subject is indicated as $B_2$. The utilization index was also unchanged in the second experiment for $B_2$ as shown in table 3.

TABLE 3

*Index of utilization[1] of crystalline ascorbic acid and an equivalent amount of vitamin C from red raspberries*

| SUBJECTS | DIETARY SUPPLEMENT | ASCORBIC ACID | | | BODY WEIGHT | UTILIZATION COEFFICIENT | $\sqrt{\text{AGE}}$ | UTILIZATION INDEX |
|---|---|---|---|---|---|---|---|---|
| | | Intake | Output | Difference | | | | |
| | | *mg.* | *mg.* | *mg.* | *kg.* | | | |
| A | Ascorbic acid | 360 | 188 | 172 | 51.26 | 3.4 | 4.8 | 16.2 |
| | Raspberries | 360 | 330 | 30 | | 0.6 | | 2.8 |
| B | (1) Ascorbic acid | 360 | 110 | 250 | 51.71 | 4.8 | 5.4 | 26.1 |
| | Raspberries | 360 | 144 | 216 | | 4.2 | | 22.5 |
| | (2) Ascorbic acid | 360 | 99 | 261 | | 5.0 | | 27.3 |
| | Raspberries | 360 | 90 | 270 | | 5.2 | | 28.3 |
| C | Ascorbic acid | 360 | 202 | 158 | 62.60 | 2.5 | 6.0 | 15.2 |
| | Raspberries | 360 | 276 | 84 | | 1.3 | | 8.0 |
| D | Ascorbic acid | 360 | 197 | 163 | 49.90 | 3.3 | 5.3 | 17.3 |
| | Raspberries | 360 | 206 | 154 | | 3.1 | | 16.5 |
| E | Ascorbic acid | 360 | 140 | 216 | 61.69 | 3.6 | 5.6 | 19.9 |
| | Raspberries | 360 | 220 | 140 | | 2.3 | | 12.8 |
| F | Ascorbic acid | 360 | 197 | 163 | 60.33 | 2.7 | 6.0 | 16.2 |
| | Raspberries | 360 | 186 | 174 | | 2.9 | | 17.3 |
| G | Ascorbic acid | 360 | 119 | 241 | 66.68 | 3.6 | 6.1 | 22.0 |
| | Raspberries | 360 | 182 | 178 | | 2.7 | | 16.2 |

[1] Index of utilization was calculated from Sendroy and Schultz, J. Clin. Invest., vol. 15, p. 369, 1936.

The data indicate that 40 mg. of ascorbic acid obtained from red raspberries were fully as well utilized as the same amount of the vitamin in crystalline form.

Short period collections of urine rather than the total 24-hour amount have been suggested by some workers as an adequate indication of whether there is a marked response to the test dose. Harris and Abbasy ('37) analyzed a 3-hour

morning specimen, whereas other workers (Goldsmith and Ellinger, '39) based their study on a 6-hour collection.

In this investigation 6-hour collections of urine were made daily for each subject; the bladder was emptied on rising in the morning and the urine for the succeeding 6 hours was collected and the vitamin C content determined immediately by titration. The per cent of the total 24-hour excretion of ascorbic acid which was eliminated in the first 6 hours was then calculated for each subject. The per cent excreted in this period varied for the different subjects and showed a wide variation for the same subject. This is illustrated by the following data for two subjects, expressed as per cent of the 24-hour excretion for the different dietary periods: subject A, preliminary period, 37, 18, 33; period I, 28, 35; period II, 47, 55, 60, 61, 45; period III, 29, 34, 52 and period IV, 39, 44, 59, 25, 33; subject D, preliminary period, 32, 25, 43; period I, 47, 44; period II, 62, 39, 54, 47, 50; period III, 27, 40, 50; period IV, 66, 56, 45, 50, 52.

The other subjects showed a similar range of excretion values; the lowest value was 6% for subject C who, on another day, excreted 61% of the total in the first 6 hours. With such variations it is to be questioned whether the 6-hour urine specimen can give reliable information regarding the total daily excretion of ascorbic acid.

## SUMMARY

Seven college women served as subjects for a study of the comparative utilization of ascorbic acid in red raspberries and in the crystalline form of the vitamin. The subjects were 'saturated' with vitamin C at the beginning of each test period and consumed weighed amounts of the same basal diet throughout.

The urinary excretion and blood plasma level of ascorbic acid were similar for the same subject when receiving vitamin C from red raspberries and from crystalline ascorbic acid. There was considerable variation among subjects with respect

to their blood level and excretion of ascorbic acid under comparable conditions.

The utilization index as calculated from Sendroy's formula showed the ascorbic acid of red raspberries to be as well utilized as crystalline ascorbic acid.

The per cent of the total 24-hour excretion of ascorbic acid eliminated during the first 6 hours varied widely in the same individual and for the different subjects.

## LITERATURE CITED

ELMBY, A., AND E. WARBURG 1937 The inadequacy of synthetic ascorbic acid as an antiscorbutic agent. Lancet, vol. 11, p. 1363.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. and Med., vol. 34, p. 146.

GOLDSMITH, G. A., AND G. F. ELLINGER 1939 Ascorbic acid in blood and urine after oral administration of a test dose of vitamin C. Arch. Int. Med., vol. 63, p. 531.

HARRIS, L. J., AND M. A. ABBASY 1937 A simplified procedure for the vitamin C urine test. Lancet, vol. 11, p. 1429.

HAUCK, H. 1938 Personal communication.

HAWLEY, E. E., D. J. STEPHENS AND G. ANDERSON 1936 The excretion of vitamin C in normal individuals following a comparable quantitative administration in the form of orange juice, cevitamic acid by mouth and cevitamic acid intravenously. J. Nutrition, vol. 11, p. 135.

MCGOVERN, T., C. E. GANNON AND I. S. WRIGHT 1939 Vitamin C deficiency—clinical and therapeutic problems. Am. J. Med. Sci., vol. 197, p. 310.

MCHENRY, E. W., AND M. GRAHAM 1935 Observations on the estimation of ascorbic acid by titration. Biochem. J., vol. 29, p. 2013.

SENDROY, JR. J., AND M. P. SCHULTZ 1936 Studies of ascorbic acid and rheumatic fever. I. Quantitative index of ascorbic acid utilization in human beings and its application to the study of rheumatic fever. J. Clin. Invest., vol. 15, p. 369.

SENDROY, JR. J. 1937 Personal communication.

# GROWTH OF RATS ON HIGH FAT AND LOW FAT DIETS, DEFICIENT IN THE ESSENTIAL UNSATURATED FATTY ACIDS [1]

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FIVE FIGURES

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During the past several years it has been shown beyond doubt that the rigorous exclusion of fat from the diet of the rat results in a typical deficiency disease, of which the outstanding symptom is subnormal growth. Since the impairment of growth can be prevented or cured only by the feeding of small amounts of either linoleic, linolenic, arachidonic, or docosahexaenoic acid, it has been concluded that these more highly unsaturated fatty acids, essential for normal growth and health, cannot be synthesized by the rat—at least in appreciable amounts—and are therefore essential dietary constituents (Hume et al., '38).

In the course of earlier studies with elaidic acid (Sinclair, '35), some young rats at weaning age were fed on a diet very rich in elaidin [3] (diet 290, table 1). This diet was identical in composition with our standard low fat diet (diet 3, table 1) save for the replacement of the sucrose by an isocaloric amount of the fat elaidin. The contrast between the growth of the rats

[1] Part of this work was presented before the American Society of Biological Chemists at Washington, D. C., in March, 1936.

[2] A considerable part of this work was done in the Department of Biochemistry and Pharmacology, University of Rochester Medical School, Rochester, N. Y.

[3] The term 'elaidin' is used to designate the solid fat prepared from olive oil by treating with $N_2O_3$ and crystallizing several times from acetone. On the basis of their iodine number, the fatty acids of elaidin are assumed to consist of about 85% elaidic acid and 15% saturated fatty acids.

on the high elaidin diet and that typical of the high carbohydrate diet was very striking. On the low fat diet (fig. 1, curve 1) growth, though subnormal, is maintained for several months and, when it finally stops, the subnormal weight is maintained more or less constant for months before the final decline sets in. On the high elaidin diet, on the other hand (fig. 1, curve 3), growth proceeded for only 4 to 6 weeks and stopped, rather abruptly, when the average weight was only about 100 gm.[4] This weight was usually maintained for several weeks and then a gradual decline set in, ending in the death of the animal at an age of about 4 months.

At this same time some young rats had been started at weaning age on a high elaidin diet (290-C, table 1) differing

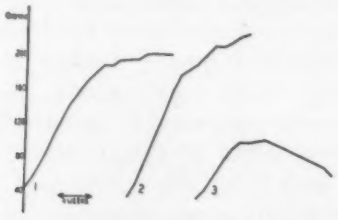


Fig. 1 Curve 1—Composite weight record of sixteen male rats fed on a high carbohydrate low fat diet (no. 3, table 1). Curve 2—Composite weight record of seven male rats fed on diet 290-C, elaidin (table 1). Curve 3—Composite weight record of five male rats fed on diet 290, elaidin (table 1).

from diet 290 in that about 5% of the elaidin was replaced by a 1:1 mixture of cod liver and corn oils. As shown by curve 2, figure 1, the growth of rats on this latter high fat diet was very good indeed. Furthermore, several rats were transferred from the straight elaidin diet to diet 290-C after there had been a considerable loss in weight, and death seemed imminent. Curve 1, figure 2, which is quite typical, shows that there is a prompt resumption of growth. Since cod liver and

[4] The maximum weight attained was found to depend largely on the weight of the rat when placed on the elaidin diet. Some animals, weighing 48 gm. when 21 days old, reached a weight of 146 gm. The amount and quality of the depot fat in the young rat appear to be of primary importance.

corn oils are rich in the essential unsaturated fatty acids, one is led to conclude that the very poor growth of the rats on the straight elaidin diet is due to a severe deficiency of those fatty acids which are essential for growth and health.

Naturally, the question arises as to the reason for the much better growth on the high carbohydrate diet. Up to the present time it has been assumed that the unsaturated fatty acids needed for the growth of rats on high carbohydrate low fat diets have been derived exclusively from two sources:

TABLE 1

*Percentage composition of diets*

| INGREDIENTS | PER 100 GM. | | |
|---|---|---|---|
| | Diet 3 | Diet 290 | Diet 290-C |
| Casein [1] | 17.1 | 28.1 | 28.1 |
| Sucrose | 70.3 | | |
| Salt mixture [2] | 3.8 | 6.3 | 6.3 |
| Yeast | 8.8 | 14.4 | 14.4 |
| Elaidin | | 51.2 | 48.4 |
| Corn oil | | | 1.4 |
| Cod liver oil [3] | | | 1.4 |

[1] Neither the casein nor the yeast was extracted with fat solvents. By analysis, the casein in diet 3 was found to supply 340 mg. and the yeast 505 mg. of total fatty material per 100 gm. of diet.

[2] McCollum, E. V., and Simmonds, N., J. Biol. Chem., vol. 33, p. 63 (1918).

[3] Vitamins A and D were supplied to rats on diet 3 or 290 in the following ways: (1) In the earlier experiments, the rats received daily the unsaponifiable material prepared from 150 mg. of cod liver oil, dissolved in 2 drops of mineral oil; (2) more recently, the rats were given 1 mg. of percomorph oil dissolved in 2 drops of mineral oil or 2 drops of melted elaidin. This method is more convenient and gives growth records indistinguishable from the first.

the small amount of fat in the diet; and the store of fat laid down in the depots during the suckling period. In the case of the rats fed on the high carbohydrate and high elaidin diets, the amount of unsaturated fatty acids derived from these two sources must be about the same. Nevertheless, the deficiency of essential fatty acids seems to be much less severe in rats on a high carbohydrate diet than in those on the high elaidin diet.

There appear to be two possible explanations. One is that the rats on the high carbohydrate diet are able to synthesize certain of the fatty acids needed for growth, even though the amount or kind—or both—synthesized is insufficient to maintain normal growth and health. In the case of the rats on the high fat diet, such synthesis of fatty acids from carbohydrate would, of course, be completely suppressed. They would therefore be entirely dependent upon the diet and pre-existing stores for the supply of essential fatty acids. The second possibility is that the requirement of essential fatty acids is greater on a high fat than on a low fat diet, either because of the intensity of fat and, therefore, of phospholipid metabolism or because of a purely physical 'masking' of the essential unsaturated fatty acids by the large amount of elaidic acid simultaneously being absorbed and transported. In either case, one would have to assume that the supply of unsaturated fatty acids in the food and stores is sufficient to permit considerable growth on the high carbohydrate diet but is quite inadequate for rats on the high fat diet.

### RESTORATION OF GROWTH

After growth had stopped, a number of rats on the high elaidin diet were given daily, by mouth, graded amounts of corn oil. Representative records are given in figure 2 (curves 2, 3, 4 and 5). It will be seen that 1 drop (20 mg.) of corn oil, or of percomorph oil,[5] is insufficient to induce a significant gain in weight although it does prevent the decline which otherwise would probably have set in. On the other hand, 5 drops (100 mg.) of corn oil induce a pronounced increase in weight; the effects of 10 and 20 drops were, in general, still better. In every case, however, growth stopped when the animal was still well below the normal adult weight.

An even more striking resumption of growth takes place if the high elaidin diet is replaced by one rich in carbohydrate.

[5] Mead Johnson & Co. Oleum percomorphum.

The five growth records in figure 3, representative of fifteen in all and including the poorest, illustrate the rapid gain in weight which promptly sets in when the elaidin diet is

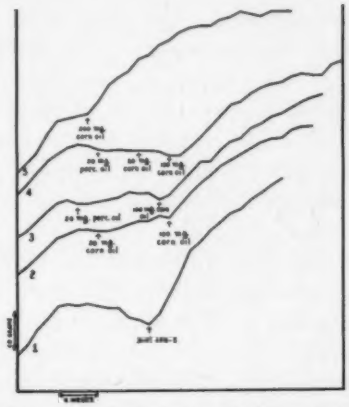

Fig. 2 Curve 1—Weight record of a male rat fed on diet 290, elaidin and then transferred to diet 290-C, elaidin. Curves 2 to 5—Weight records of rats fed on diet 290, elaidin supplemented by percomorph oil or corn oil.

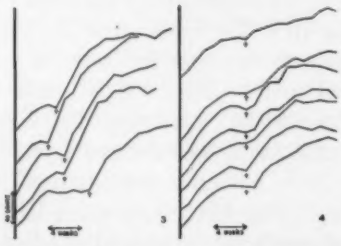

Fig. 3 Weight records of rats fed on diet 290, elaidin and then changed to diet 3 (table 1).

Fig. 4 Weight records of rats fed on diet 290, elaidin and then fed on a high carbohydrate diet very low in fat (diet 6 b).

replaced by diet 3. The only change in the diet is the replacement of the fat by sucrose. After a gain of 80 gm. or more, resulting in some cases in a doubling of the weight in 4 weeks, the growth ceases once more when the weight is about

the same as that characteristic of rats fed from weaning age on the same high carbohydrate diet.

When these results were first obtained, it was felt that the only satisfactory interpretation was that the resumption of growth on the carbohydrate diet had been made possible by the synthesis of the necessary fatty acids from carbohydrate (Sinclair, '36). Later it was recognized that the restored growth could conceivably be attributed to the small amount of essential acids, present in the casein and yeast fat, which, though quite unable to support growth on the elaidin diet, became effective when the fat was replaced by sucrose.

It was decided therefore to repeat the experiments, using a diet which, though not strictly fat-free, contained very much less fat than diet 3. The composition of this diet (no. 6 b) was as follows: Vitamin-free casein,[6] 16.6%; sucrose, 75.6%; salt mixture, 3.8%; Harris water-soluble yeast concentrate,[7] 4%. Vitamins A and D were supplied by giving daily 2 drops of propylene glycol carrying 50 γ of carotene [8] and 0.2 γ of calciferol.[9]

As in the earlier experiments, young rats were fed on the elaidin-containing diet 290 until growth had ceased and the weight had remained constant for several weeks.[10] The diet was then changed to 6 b. The weight records of seven rats are shown in figure 4. In every case there was a resumption of growth. The gain in weight ranged from 34 gm. to 60 gm., the maximum gain in a single week being 19 gm.

Now, analysis of the Harris yeast concentrate, the only possible source of essential fatty acids in diet 6 b, showed that 10 gm. contained 28 mg. of ether-soluble and petroleum ether-soluble material. One may assume that perhaps 25% of these fatty acids consists of linoleic acid (Newman and

[6] Obtained from S. M. A. Corporation, Cleveland, O.

[7] Obtained from Harris Laboratories, Tuckahoe, N. Y.

[8] Kindly donated by Hoffmann-LaRoche, Ltd.

[9] Kindly supplied by Dr. C. E. Bills of Mead Johnson & Co.

[10] In all cases, the rats were kept in individual cages with bottoms made of wire screen (¼ inch mesh).

Anderson, '33). On the basis of a daily consumption of 10 gm. of ration per day, each rat would thus obtain in the diet 1.1 mg. of fatty acid and about 0.3 mg. of linoleic acid.

It is probably safe to assume, on the basis of existing data (Sinclair, '30), that an increase of 10 gm. in the body weight of a rat involves an increase of about 100 mg. in the phospholipid fatty acid content of the body. Consequently it may be calculated that a rat which increased its weight by 19 gm. in a week probably added about 190 mg. to its phospholipid fatty acids and, during the same period, took in not more than 8 mg. of fatty acids with its food. Clearly therefore, the daily increment in phospholipid fatty acids is several times greater than the amount of fatty acid that could possibly have been obtained from the diet. Because of the nature of the previous diet, the depot fat of these rats can conceivably be a source only of some of the fully saturated acids, of elaidic acid, and possibly of some oleic acid. From what is known about the phospholipids, it is very unlikely that these fatty acids, together with the trace of linoleic obtained from the diet, would suffice for phospholipid synthesis. One is led to conclude therefore that the rats on the high carbohydrate diet synthesized the bulk of the fatty acids needed for growth. Whether or not even the limited growth which did occur was made possible only by reason of the trace of fat present in the diet is, of course, a question which can only be settled by feeding a strictly fat free diet.

On comparison of the growth curves in figures 4 and 3, it is evident that the gain in weight by rats fed on the more highly purified diet 6 b was inferior to that of most of the rats on diet 3. To rule out the possibility of there being an insufficiency of thiamin and lactoflavin, 10 $\gamma$ of each of these two factors were fed daily for several weeks to the rats on diet 6 b. No effect on growth was apparent. It seems likely therefore that the difference is due to the higher fat content of diet 3. This is borne out by the growth records in figure 5 which show that either the replacement of diet 6—similar to 6 b

except for the use of non-extracted casein—by diet 3 or the feeding of 50 mg. of yeast fat caused a resumption of growth.

An increase or a decrease in body weight has been the only feature dealt with in this paper. It needs to be emphasized that, even though the rats gained considerably in weight on the high carbohydrate diet, their condition did not improve to an extent at all comparable to that of the animals fed a little corn oil in addition to the elaidin diet. Indeed, soon after growth had ceased once more, the weight began to decline and, within a short time, severe hematuria and other symptoms developed.

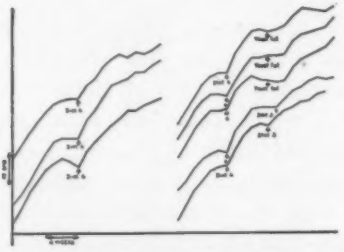

Fig. 5 Weight records of rats fed on diet 290, elaidin until growth had ceased and then transferred to a high carbohydrate diet low in fat (diet 6). When growth had stopped once more, some were transferred to diet 3; others were fed 50 mg. of yeast fat.

## DISCUSSION

The findings presented in this paper can best be explained on the basis of the assumption that the rat is capable of synthesizing from carbohydrate the bulk of the fatty acids needed for growth. On the high elaidin diet, the supply of essential fatty acids derived from the pre-existing fat stores is sufficient to permit moderately good growth for a time; the greater the supply, the better the growth. When the supply is exhausted, growth stops because the small amount of essential fatty acid present in the diet is quite inadequate. If sufficient corn oil is fed, growth is restored. Twenty milligrams of corn oil is however insufficient, even though, on a high carbo-

hydrate diet, such an amount, judging from other evidence (Mackenzie et al., '39) permits excellent growth. The fatty acids synthesized from carbohydrate make up the deficiency. On replacing the elaidin of the diet by sucrose, after growth has stopped, synthesis of fatty acids sets in and these fatty acids, at least with the trace present in the diet, are sufficient to permit rapid and extensive growth. If the rat is placed on the high carbohydrate diet at weaning age, the synthesized fatty acids, together with those in the depots and in the diet, permit growth up to about 70% of the average normal weight. Normal growth and health can, however, only be secured by feeding certain of the unsaturated fatty acids.

On the other hand, it is entirely possible that an increase in the requirement of essential unsaturated fatty acids as a result of the very high fat diet is, in part at least, responsible for the poor growth and early decline of rats on the elaidin diet and for most of the observations reported above.

Regardless of which is the correct explanation, it ought to be possible to duplicate all of the results presented in this paper with some fat other than elaidin, provided it is totally free from the essential unsaturated fatty acids. Some experiments were carried out with hydrogenated coconut oil. In general, the results obtained were the same as those above. Evans and Lepkovsky ('32) some years ago reported very poor growth, comparable to that on elaidin, on diets rich in the glycerides of the completely saturated acids of coconut oil. There is no reason to believe that the growth of rats on the diet rich in elaidin is in any sense characteristic of that fat alone.

## SUMMARY

On a diet of casein, salt mixture, dried yeast and the fat, elaidin, supplemented with vitamins A and D, rats cease growing when only about 100 gm. in weight. After several weeks at constant weight they go into a decline and die. Since growth and health are readily restored by feeding a little corn oil, the impairment in growth is attributed to a severe deficiency of the essential unsaturated fatty acids.

Replacement of the elaidin by sucrose also results in a rapid and extensive gain in weight. A similar, though smaller, gain occurs when the elaidin diet is replaced by one rich in carbohydrate and very poor in fat.

It is concluded that the better growth of rats on a high carbohydrate than on a high fat diet, both equally poor in essential fatty acids, is due, in part at least, to the synthesis of the fatty acids necessary for growth. An increase in the requirement of essential fatty acids by rats on a high fat diet may also contribute to the poor growth obtained.

## LITERATURE CITED

EVANS, H. M., AND S. LEPKOVSKY 1932 Vital need of the body for certain unsaturated fatty acids. II. Experiments with high fat diets in which saturated fatty acids furnish the sole source of energy. J. Biol. Chem., vol. 96, p. 157.

HUME, E. M., L. C. A. NUNN AND I. SMEDLEY-MACLEAN 1938 Studies of the essential unsaturated fatty acids in their relation to the fat-deficiency disease of rats. Biochem. J., vol. 32, p. 2162.

NEWMAN, M. S., AND R. J. ANDERSON 1933 The chemistry of the lipids of yeast. I. The composition of the acetone-soluble fat. J. Biol. Chem., vol. 102, p. 219.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low fat diet. Biochem. J., vol. 33, p. 935.

SINCLAIR, R. G. 1930 The metabolism of the phospholipids. II. The influence of growth on the phospholipid (and cholesterol) content of the white rat. J. Biol. Chem., vol. 88, p. 575.

———— 1935 The metabolism of the phospholipids. VIII. The passage of elaidic acid into tissue phospholipids. Evidence of the intermediary role of liver phospholipid in fat metabolism. J. Biol. Chem., vol. 111, p. 515.

———— 1936 Evidence of the synthesis of essential fatty acids by the rat. J. Biol. Chem., vol. 114, p. XCIV.

# THE INDIGESTIBLE CARBOHYDRATES OF FEEDS

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The indigestible carbohydrate portion of feeds, especially of those referred to as roughages, has always been of considerable interest to nutrition workers. For over 75 years this indigestible residue has been referred to as crude fiber. The official A.O.A.C. method for its determination, known as the Weede method, has always been recognized as purely empirical, having little correlation with the residues from the true digestive processes, and failing to give a true conception of the composition of the residue. Therefore, we have no way of estimating the relative digestibility of the quantity of crude fiber reported, as the chemical makeup of this material may be quite different in samples from various plants, and the utilization entirely different depending upon the lignin-cellulose ratio. These difficulties have always been admitted but only in recent years has any systematic attempt been made to break down this material by more accurate methods of analysis for lignin, hemicellulose, and cellulose separately. A comprehensive study made during the past 2 years in this laboratory has demonstrated that none of the methods is entirely satisfactory, and, also, that one cannot expect to recover 100% of the original residue by purely empirical procedures.

It is much easier to cite reasons for these discrepancies than to offer remedies. In the first place, although numerous and extensive studies have been made, the true structure and properties of cellulose, lignin, or hemicellulose are not known,

and, therefore, only empirical methods are used in the determinations. Furthermore, the nature of the balance of the feed may affect profoundly the results obtained. These facts are suggested in the reports of many investigators. The excellent reviews as well as the original work of Norman ('34 and '35), and of Norman and Jenkins ('34) demonstrate clearly the difficulties presented.

When one attempts to recover the various fractions from a single sample, the task becomes still more difficult. Even the methods used are open to criticism in certain instances. In the method of Williams and Olmsted ('35) the use of an alkaline-enzyme digest results in the loss of some of the hemicellulose, and, in certain cases, separate determinations must be made for its complete recovery. The use of 60% $H_2SO_4$, in the light of the work of many investigators, notably Ritter, Seborg and Mitchell ('32), and Sherrard and Harris ('32) does not give as good results as a 70 to 72% solution. Under the conditions outlined this procedure is open to criticism. First, with 60% sulfuric acid it is doubtful if all the cellulose is entirely removed; second, an investigation of the literature does not present evidence that uronic acid is converted quantitatively into pentoses, as stated; and third, there is evidence that pentoses would be partly destroyed in the long acid hydrolysis.

In the Crampton and Maynard method ('38), on the other hand, the use of formaldehyde in the determination of lignin is not accepted by many lignin chemists due to the fact that formaldehyde condensation products must surely yield a high lignin result, and no measure of the hemicellulose fraction is presented. Although no method can be entirely quantitative until more fundamental methods are perfected, nevertheless, the rather empirical procedure as outlined does present a much more complete picture of the true composition of feeds than the crude fiber as ordinarily reported.

In view of these considerations, the modification of the Williams and Olmsted procedure has been used in analyzing many common cereal grains and forage crops as well as feces samples, and has proved to be the most acceptable in com-

parative tests. In digestibility studies attempts were made to ascertain if certain fractions of this material might be more digestible than others by comparing the relative proportions as the test substance passed through the intestinal tract of sheep and cattle. The ratio between the quantities of lignin, cellulose, and hemicellulose might be quite a changeable one if hemicellulose proved to be more digestible than lignin, and the so-called crude fiber content of certain feeds more desirable than an equal amount in plants having a different composition.

## EXPERIMENTAL PART

Samples of cereal grains, seeds, hays, prairie grasses, and mixed feeds produced and commonly used in this state were chosen from samples being analyzed in this station. The samples were dried, ground in a Wiley mill, and further powdered in a Merker mill until they would completely pass through a 60-mesh sieve. They were then redried, and 0.5 gm. samples weighed in duplicate. The method of analysis was a somewhat modified form of the Williams-Olmsted procedure, and is briefly described as follows:

The sample was first extracted 16 hours with ether, dried, and transferred to a 250 ml. Erlenmeyer flask, and covered with a 50 ml. beaker. Twenty-five milliliters of water were added, and the flask and contents sterilized for 2 hours at 20 pounds pressure. The flasks were cooled, and the enzyme-digest mixture added which consisted of 20 ml. of bile buffer, 5 ml. of the pancreatin-sodium chloride solution, and a few drops of toluene. The flasks were transferred to a constant temperature water bath controlled at 45°C., and left for 72 hours with frequent shaking. At the end of this period the contents of the flasks were filtered through a silk bolting cloth, washed with hot alcohol, benzene, alcohol, and ether. The residue was then transferred to the original flask, and treated with 20 ml. of cold 70% sulfuric acid, and kept with frequent shaking for 16 hours at 4 to 10°C., as suggested in the procedure of Sherrard and Harris ('32). The residue and acid

were then transferred to 1000 ml. tall, lipless beakers, treated with 450 ml. of water, and boiled for 3 hours under condensers, and at once filtered with suction through RA 98, 25 ml. alundum crucibles. The filtrate was then transferred to 500 ml. volumetric flasks. The alundum crucibles containing the lignin were first washed very thoroughly with hot water to remove acids and sugars, then with hot water, alcohol, benzene, and ether, then dried, weighed, ashed, and reweighed, and the loss of weight calculated as lignin. The filtrate was exactly neutralized with 50% NaOH, and diluted to 500 ml. Five milliliter aliquots were used to determine the reducing sugars, and similar quantities of the samples which had been previously fermented with well-washed yeast were used for non-fermentable sugars following the original technique. From these determinations cellulose and hemicellulose were calculated as directed. The reducing sugar values were determined by comparing the titrations with curves which had been prepared from known sugars dissolved in similar solvents. There is nothing forbidding about the procedure. It is long and requires attention at definite intervals. Great care, skill, and attention as to the exact pH, time, and temperature were found to be necessary; however, with sufficient experience it can become a regular routine procedure, and can easily be carried out by any experienced technician. For the preparation of reagents and details of procedure one is referred to the Williams-Olmsted article ('35), and for questions concerning the sugar determination and preparation of sugar curves reference is made to the original Shaffer-Somogyi method ('33). Samples were also analyzed for crude fiber by the usual A.O.A.C. methods. The data for a number of common Oklahoma hays and grasses are recorded in table 1, and similar data for some common food concentrates are given in table 2. These data demonstrate that no fixed ratio exists between either the amounts of lignin, cellulose, and hemicellulose or crude fiber and the total indigestible residue of cereal grains.

STUDY OF INTESTINAL CONTENTS

A further purpose of this study was to determine if crude fiber from various sources was equally well utilized by animals; that is, to determine if a sample composed largely of hemicellulose or cellulose might have a higher coefficient of digestibility than one composed largely of lignin. It was possible to secure these data, as coefficients of digestibility of various feeds were being determined by this and cooperating

TABLE 1

*Lignin, cellulose, and hemicellulose content of hay and related plant products. Milligrams per gram sample*

| SAMPLE | CRUDE FIBER | LIGNIN | HEMICELLULOSE | CELLULOSE | TOTAL INDIGESTIBLE RESIDUE |
|---|---|---|---|---|---|
| Alfalfa hay | 246 | 180 | 112 | 153 | 445 |
| Alfalfa leaf meal | 166 | 129 | 79 | 131 | 339 |
| Bermuda grass | 254 | 111 | 158 | 192 | 461 |
| Johnson grass | 329 | 142 | 91 | 88 | 321 |
| Little blue stem | 324 | 199 | 156 | 152 | 507 |
| Switch grass | 320 | 217 | 139 | 102 | 458 |
| Atlas sorgo | 189 | 162 | 94 | 133 | 389 |
| Clover hay | 213 | 218 | 49 | 62 | 329 |
| Sweet clover | 305 | 209 | 99 | 105 | 413 |
| Dwarf yellow milo | 206 | 153 | 96 | 69 | 318 |
| Millet hay | 276 | 179 | 121 | 109 | 409 |
| Mung bean hay | 220 | 163 | 95 | 92 | 350 |
| Soy bean hay | 151 | 330 | 158 | 125 | 613 |
| Wheat straw | 364 | 310 | 146 | 115 | 571 |
| Timothy hay | 303 | 155 | 188 | 169 | 512 |

departments at the same time. Rats, chickens, sheep, and cattle were used in these experiments. The data for rats and sheep are presented. The usual methods of procedure for metabolism experiments were followed by the use of specially constructed feeders and cages with perforated floors which permitted the measurement of the weight of feed and excreta daily. These tests have been repeated a number of times, and the averages of a series of analyses of the data for digestibility coefficients of lignin, cellulose, and hemicellulose are

TABLE 2

*Lignin, cellulose, and hemicellulose content of grains and seed concentrates. Milligrams per gram sample*

| SAMPLE | CRUDE FIBER | LIGNIN | HEMI-CELLULOSE | CELLULOSE | TOTAL INDIGESTIBLE RESIDUE |
|---|---|---|---|---|---|
| Barley | 54 | 68 | 59 | 26 | 154 |
| Buck wheat | 105 | 139 | 92 | 42 | 273 |
| Broom corn | 84 | 83 | 38 | 31 | 152 |
| Yellow corn | 21 | 23 | 49 | 45 | 117 |
| Flint corn | 20 | 73 | 62 | 37 | 172 |
| Cottonseed meal | 115 | 93 | 89 | 75 | 257 |
| Cottonseed burrs | 322 | 195 | 84 | 101 | 380 |
| Flaxseed | 62 | 86 | 52 | 18 | 156 |
| Kafir | 19 | 39 | 45 | 38 | 122 |
| Millet | 41 | 160 | 93 | 97 | 350 |
| Milo | 22 | 56 | 45 | 32 | 133 |
| Oats | 111 | 60 | 103 | 79 | 332 |
| Oat hulls | 300 | 160 | 242 | 213 | 615 |
| Peanut hulls | 563 | 448 | 127 | 162 | 737 |
| Wheat | 26 | 39 | 58 | 40 | 137 |
| Wheat shorts | 56 | 73 | 89 | 62 | 225 |
| Wheat bran | 89 | 150 | 155 | 68 | 373 |
| Rye | 22 | 43 | 56 | 38 | 138 |
| Timothy seed | 33 | 46 | 41 | 34 | 121 |
| Vetch seed | 56 | 66 | 49 | 58 | 73 |
| Soy beans | 50 | 88 | 66 | 26 | 180 |
| Cow peas | 51 | 92 | 48 | 54 | 194 |
| Field peas | 48 | 178 | 51 | 50 | 279 |
| Navy beans | 50 | 55 | 60 | 64 | 180 |
| Garden peas | 37 | 94 | 47 | 61 | 202 |
| Lima beans | 36 | 21 | 66 | 42 | 129 |
| Rice bran | 128 | 92 | 70 | 41 | 203 |
| Stringless beans | 30 | 98 | 59 | 60 | 217 |
| Serval | 359 | 408 | 173 | 148 | 729 |

presented in table 3. The basic ration was a well-balanced cereal grain mixture fortified with protein, vitamins, and minerals. To this mixture had been added finely powdered wheat straw or peanut hulls as indicated in the table. An examination of the table indicates that the amount of indigestible residue is far greater than the crude fiber, and that there is no relation between the various fractions composing this matter.

A similar study was made of the digestibility of mixtures of feed consumed by sheep. Two rations were prepared, the

first of alfalfa, corn, and molasses, the second of alfalfa, oats, and molasses. Previous utilization studies had demonstrated that the oats ration was superior to that of corn (unpublished data). This same material, analyzed according to the new procedure, and presented in table 4, fails to indicate that the fiber fractions were responsible for the more favorable development.

TABLE 3

*Apparent utilization of lignin, cellulose, and hemicellulose of mixed rat feeds*

| | AMOUNT | MILLIGRAMS PER GRAM SAMPLE AND PERCENTAGE UTILIZATION | | | | |
|---|---|---|---|---|---|---|
| | | Crude fiber | Lignin | Hemicellulose | Cellulose | Total indigestible residue |
| | gm. | | | | | |
| Basic ration | 116 | 38 | 26 | 37 | 27 | 90 |
| Feces | 28 | 147 | 93 | 134 | 82 | 310 |
| Coefficient of utilization | | 8% | 13% | 12% | 26% | 16% |
| Basic ration plus 15% peanut hulls | 114 | 116 | 60 | 42 | 99 | 201 |
| Feces | 31 | 332 | 189 | 109 | 87 | 386 |
| Coefficient of utilization | | 20% | 13% | 27% | 75% | 49% |
| Basic ration plus 30% peanut hulls | 117 | 217 | 116 | 69 | 104 | 289 |
| Feces | 47 | 434 | 239 | 122 | 97 | 488 |
| Coefficient of utilization | | 19% | 18% | 29% | 62% | 32% |
| Basic ration plus 20% wheat straw | 90 | 106 | 55 | 62 | 111 | 228 |
| Feces | 25 | 290 | 171 | 169 | 94 | 435 |
| Coefficient of utilization | | 24% | 6% | 23% | 76% | 45% |
| Basic ration plus 30% wheat straw | 100 | 118 | 92 | 85 | 73 | 251 |
| Feces | 25 | 313 | 284 | 165 | 103 | 552 |
| Coefficient of utilization | | 31% | 9% | 50% | 73% | 52% |

At the completion of the experiment some of the animals were sacrificed, and samples from indicated levels of the stomach and intestines were similarly analyzed. The purpose of these tests was to ascertain if the ratio between the percentages of lignin, hemicellulose, and cellulose remained the same throughout the digestive tract. The results are tabulated in table 5, and the actual amount of each constituent is also

calculated in terms of its percentage of the remaining indigestible residue. An increase in the percent of lignin, for instance, in food passing through the intestine, indicates that it is being utilized less than one of the other two components.

TABLE 4

*Utilization of cellulose, lignin, and hemicellulose of mixed feeds by sheep*

| SAMPLE | GRAMS | MILLIGRAMS PER GRAM SAMPLE | | | | |
|---|---|---|---|---|---|---|
| | | Fiber | Lignin | Hemicellulose | Cellulose | Total indigestible residue |
| Molasses | 230 | 0 | 0 | 0 | 0 | 0 |
| Alfalfa | 454 | 255 | 180 | 112 | 153 | 445 |
| Corn | 230 | 19 | 23 | 49 | 45 | 117 |
| Feces | 239 | 328 | 183 | 128 | 163 | 476 |
| Coefficient of utilization | | 34.7 | 49.3 | 40.8 | 50.7 | 50.1 |
| Molasses | 230 | 0 | 0 | 0 | 0 | 0 |
| Alfalfa | 454 | 255 | 180 | 112 | 153 | 445 |
| Oats | 230 | 143 | 60 | 103 | 79 | 332 |
| Feces | 245 | 382 | 318 | 140 | 152 | 610 |
| Coefficient of utilization | | 36.9 | 18.4 | 42.9 | 57.4 | 46.0 |

TABLE 5

*Lignin, cellulose, and hemicellulose of intestinal content of sheep*

| | MILLIGRAMS PER GRAM SAMPLE | | | | | PER CENT OF TOTAL RESIDUE | | |
|---|---|---|---|---|---|---|---|---|
| | Crude fiber | Lignin | Hemicellulose | Cellulose | Total indigestible residue | Lignin | Hemicellulose | Cellulose |
| *Corn ration* | | | | | | | | |
| Rumen | 338 | 152 | 140 | 197 | 489 | 31 | 29 | 40 |
| Reticulum | 310 | 167 | 117 | 179 | 463 | 36 | 25 | 39 |
| Abomasum | 284 | 154 | 117 | 167 | 438 | 35 | 27 | 38 |
| Omasum | 330 | 160 | 108 | 184 | 452 | 35 | 24 | 41 |
| Ileum | 103 | 62 | 45 | 59 | 166 | 37 | 27 | 36 |
| Ascending colon | 301 | 170 | 123 | 161 | 454 | 37 | 27 | 36 |
| Sigmoid colon | 328 | 183 | 133 | 166 | 482 | 38 | 28 | 34 |
| *Oats ration* | | | | | | | | |
| Rumen | 217 | 230 | 97 | 66 | 393 | 58 | 25 | 17 |
| Reticulum | 249 | 315 | 98 | 97 | 510 | 62 | 19 | 19 |
| Abomasum | 237 | 281 | 95 | 119 | 495 | 57 | 19 | 24 |
| Omasum | 218 | 332 | 78 | 89 | 499 | 66 | 16 | 18 |
| Ileum | 142 | 207 | 66 | 83 | 356 | 58 | 19 | 23 |
| Ascending colon | 371 | 305 | 138 | 161 | 605 | 50 | 23 | 27 |
| Sigmoid colon | 383 | 318 | 148 | 154 | 620 | 51 | 24 | 24 |

## CONCLUSIONS

1. A modified method of determining lignin, hemicellulose, and cellulose of feeds is presented.

2. Both the total content of and the ratio between the quantities of lignin, hemicellulose, and cellulose of various cereal grains and grasses vary with the type of the material.

3. The indigestible residue determined by this method is much greater than the analogous crude fiber value.

4. The apparent utilization of cellulose in feeds is greater than that of lignin.

5. The cellulose of certain mixtures of feeds is better utilized than that of others; the utilization is somewhat determined by other ingredients of the mixture.

## LITERATURE CITED

CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition, vol. 15, p. 383.

NORMAN, A. G. 1935 The composition of crude fibre. J. Agr. Sci., vol. 25, p. 529.

NORMAN, A. G., AND S. H. JENKINS 1934 The determination of lignin. I. Errors introduced by the presence of certain carbohydrates. Biochem. J., vol. 28, p. 2147.

———— 1934 The determination of lignin. II. Errors introduced by the presence of proteins. Biochem. J., vol. 28, p. 2160.

RITTER, G. J., R. M. SEBORG AND R. J. MITCHELL 1932 Factors affecting quantitative determination of lignin with 72% $H_2SO_4$. Ind. and Eng. Chem., Analytical Edition, vol. 4, p. 202.

SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determination. J. Biol. Chem., vol. 100, p. 695.

SHERRARD, E. C., AND E. E. HARRIS 1932 Factors influencing properties of isolated wood lignin. Ind. and Eng. Chem., vol. 24, p. 103.

WILLIAMS, R. D., AND W. H. OLMSTED 1935 A biochemical method for determining indigestive residue (crude fiber) in feces: Lignin, cellulose, and non-water-soluble hemicelluloses. J. Biol. Chem., vol. 108, p. 653.

# THE INFLUENCE OF UREA INGESTION ON THE NITROGEN BALANCE AND ENERGY METABOLISM OF RATS [1]

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A survey of the literature dealing with the question of utilization of dietary urea by animals reveals that while some of the reports are conflicting (Krebs, '37), the preponderance of the more recent evidence is to the effect that urea can be at least partially utilized as a substitute for protein by polygastric animals, presumably by the aid of microorganisms. Positive results have been reported for sheep and cows (Honcamp and Koudela, '27; Schmidt et al., '37), for lambs (Sauer, '38), and for growing calves (Fingerling et al., '37; Bartlett and Cotton, '38; Hart et al., '38). The results reported for non-ruminants suggest the possibility of a species difference, but they are inconclusive.

Taylor and Ringer ('13) found that urea administered by mouth to dogs was eliminated quantitatively in the urine. In controlled feeding experiments with hogs Piepenbrock ('27) observed that the substitution of from 30 to 40% of the total protein of the ration by urea did not impair the gain in weight, thus indicating some utilization of urea by this species of animals. Moore et al. ('31) fed large quantities of urea to healthy human subjects, as part of their diet, and found that a substantial portion of the administered nitrogen was not recovered in the urine and feces. In these experiments the dietary

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nitrogen was estimated from standard food tables. On the contrary, urea fed as the only source of protein in the diet to a human subject was found to be completely recovered as such in the urine by Kocher and Torbert ('32). There appears to be no record of experiments on this phase of the problem with rats.

A number of studies directed to the determination of the energy expenditure involved in the process of urea excretion are on record, and these are directly concerned with the effect of ingestion of urea on the total energy metabolism.

Using dogs as experimental subjects Tangl ('11) obtained only a slight increase in metabolism as a result of administration of urea by stomach. Lusk ('12) and Grafe ('15) found that the administration of urea to dogs had no effect on the heat production. In contrast to these observations, Lublin ('28) later reported that the administration of urea to dogs caused an increase in energy metabolism.

In experiments with human subjects, Borsook and Winegarden ('31 a, b, c) have observed a considerable increase in energy metabolism as a result of oral administration of urea. These results were questioned by Eaton, Cordill and Gouaux ('35) who have reported experimental data showing that ingestion of urea by human subjects had no effect on the heat production. Additional evidence against such an effect of urea in human beings is found in the recent work of Carpenter ('38).

Rajzman ('36) has found no rise in energy exchange after intraperitoneal injection of urea into rabbits and rats.

The purpose of the present investigation was twofold: first, in connection with an investigation of the general problem of specific dynamic effects of proteins and amino acids, it was desired to obtain, by the use of the same general technic, direct information on the controversial question of whether the excretion of urea exerts a specific dynamic effect; and, second, it was designed to show whether rats can utilize orally administered urea as a source of protein in the body.

## EXPERIMENTAL PART

Six male albino rats weighing approximately 200 gm. each served as the experimental subjects. The rats were kept in individual cages and were subjected to the following dietary treatments during the periods and in the order indicated: (1) 8 gm. per day of a basal ration consisting of 93.7% of an approximately complete calf meal (Forbes, Kriss and Miller, '34) and 6.3% butterfat, for a period of 2 weeks, (2) 8 gm. of basal ration plus 2 gm. of urea per day, for a period of 8 days, (3) 8 gm. of basal ration for a period of 7 days, (4) 8 gm. of basal ration for 7 days followed by a fasting period of 31 hours. The food was offered in two equal portions, one at about 8 A.M. and the other at about 5 P.M.

The basal diet was of the following chemical composition: moisture, 10.3%; nitrogen, 3.25%; carbon, 42.26%; and energy, 4306 calories per gram. The analysis of the urea was as follows: moisture, 0.39%; nitrogen, 46.31%; carbon, 19.99%; and energy, 2514 calories per gram.

Urine and feces were collected from each rat during the last 7 days of the first period of feeding on the basal ration, and during the last 5 days on the urea supplemented diet. A small quantity of sodium fluoride was added to the urine as a preservative. This proved to be efficient, since determinations of nitrogen of urine preserved with sodium fluoride were found to agree well with those of urine collected in sulfuric acid from the same rats under identical dietary conditions.

The urine of each rat was analyzed for nitrogen and carbon. Energy was determined in a composite sample. The feces were first dried in an oven at approximately 60°C. and then exposed to the room air and allowed to come into equilibrium with the moisture of the air. After the weight of air-dried material for each rat was determined, the feces of all rats were combined and analyzed for nitrogen, carbon and energy.

At the end of each feeding period the rats were subjected to measurements of the respiratory exchange. These measurements began a few minutes after the rats had consumed their morning meal and continued for 7 consecutive hours. Finally,

the fasting metabolism was determined in a 7-hour period starting 24 hours after the last meal (of the basal ration) was fed to the animals. The measurements of the fasting metabolism entered into the computation of corrections of the total heat production for differences in body weight.

The apparatus used for measuring the respiratory exchange, the device for recording the movements of the animal in the respiration chamber, and the general technic employed were as described in a previous publication (Kriss, '38).

The protein metabolism was accounted for in the computation of the total metabolism in all feeding periods on the basis of the urinary nitrogen excretion in the basal period. The justification for this will become obvious from a consideration of the results of urinary analyses. The respiration and energy factors used in this computation were as determined by Kriss and Voris ('37) for beef muscle protein.

For comparative purposes the heat production was computed to a basis of uniform empty weight. The empty weights of the experimental subjects on the different dietary treatments were calculated from the total body weights on the basis of direct determinations of the alimentary fill of a separate group of rats of comparable size subjected to the same dietary treatments.

## RESULTS

The first indication that urea was not utilized by the rats to any significant extent for the building of body tissue was shown by the very slight changes in body weight resulting from the addition to the basal ration of 2 gm. of urea per day for a period of 8 days. A comparison of the weights of the rats at the end of this period with the weights attained at the end of the preceding basal period showed that the feeding of urea resulted in a slight loss in weight (from 1 to 7 gm.) in five of the six rats, and in a slight gain (2 gm.) in only one rat. Likewise, the omission of urea from the diet did not appear to affect the body weights to a significant extent.

More definite information regarding the question of utilization of urea is obtained from the chemical analysis of the urine (table 1) and from the balances of nitrogen, carbon and energy (table 2).

TABLE 1
*Urinary analysis*

| | RAT NO. | | | | | | AVERAGE |
|---|---|---|---|---|---|---|---|
| | 31 | 32 | 33 | 34 | 35 | 36 | |
| Urine from basal ration | | | | | | | |
| Nitrogen per day, mg. | 156 | 167 | 168 | 175 | 178 | 159 | 167 |
| Carbon per day, mg. | 139 | 140 | 135 | 142 | 143 | 130 | 138 |
| C: N ratio | 0.89 | 0.84 | 0.80 | 0.81 | 0.80 | 0.82 | 0.83 |
| Energy per day, calories | | | | | | | 1397 |
| Urine from basal ration plus 2 gm. urea | | | | | | | |
| Nitrogen per day, mg. | 1015 | 1078 | 1045 | 1065 | 1047 | 1056 | 1051 |
| Carbon per day, mg. | 516 | 541 | 526 | 532 | 538 | 539 | 532 |
| C: N ratio | 0.51 | 0.50 | 0.50 | 0.50 | 0.51 | 0.51 | 0.51 |
| Energy per day, calories | | | | | | | 6414 |
| Differences due to urea | | | | | | | |
| Nitrogen, mg. | 859 | 911 | 877 | 890 | 869 | 897 | 884 |
| Carbon, mg. | 377 | 401 | 391 | 390 | 395 | 409 | 394 |
| C: N ratio | 0.44 | 0.44 | 0.45 | 0.44 | 0.46 | 0.46 | 0.45 |
| Energy, calories | | | | | | | 5017 |
| Energy: N ratio | | | | | | | 5.68 |

TABLE 2
*Utilization of dietary urea by rats*

| CONSTITUENTS | DAILY INTAKE (2.0 GM. UREA) | LOST IN FECES | RECOVERED FROM URINE | | BODY BALANCES |
|---|---|---|---|---|---|
| | | | *Total* | *Per cent of intake* | *Per cent of intake* |
| Nitrogen, mg. | 926 | 7 | 884 | 95.5 | +3.8 |
| Carbon, mg. | 400 | 3* | 394 | 98.5 | +0.8 |
| Energy, calories | 5028 | 38* | 5017 | 99.8 | —0.5 |

* Calculated from the fecal nitrogen. The determined value for carbon was 18 mg., and that for energy 208 calories.

The average C: N ratio of the urine obtained when the basal ration was fed was 0.83: 1. The addition of 2 gm. of urea per day to the basal ration resulted in the lowering of the urinary C: N ratio to 0.51: 1, the urinary residues from the ingested

urea having an apparent C:N ratio of 0.45:1. This ratio is almost identical with the C:N ratio of urea (0.43:1). The consistent character of the results of the individual animals is highly significant in this relation and these results indicate that the increased excretion of urinary nitrogen caused by the ingestion of urea was practically all in the form of urea. This is substantiated by the relation of energy to nitrogen found in the urine.

The effects of ingestion of urea on the daily balances of nitrogen, carbon and energy are summarized in table 2.

Of the total nitrogen ingested as urea 95.5% was recovered from the urine and 0.7% from the feces, leaving a positive balance of only 3.8%. It is unnecessary to assume that this small balance of nitrogen was in the form of protein.

Of the total intake of carbon and energy in the form of urea 98.5% and 99.8%, respectively, were found in the urine. If the small quantity of nitrogen lost in the feces be taken to represent urea, the carbon of the urine and feces could account for 99.2% of the carbon of urea fed, and the energy of the feces and urine would exceed the energy intake as urea by 0.5%. These small balances can be considered to be well within the limits of experimental error.

In a previous study (Kriss, '38), it was found that when different proteins (casein, gelatin and heart muscle) were fed to rats as supplements to a basal ration of the kind used in these experiments, considerable proportions of the nitrogen intake were retained in the bodies. When the proteins were fed in quantities of 1.5 gm. per day, the average percentages of their nitrogen retained were, for casein 6.0%, for gelatin 13.9%, and for heart muscle 29.1%. When these proteins were fed in quantities of 3.0 gm. per day the percentages of their nitrogen retained were, on the average, 17.2% for casein, 21.5% for gelatin, and 28.6% for heart muscle. In the light of these observations, and in consideration of the fact that the rats used in the present experiments were immature, as in the previous study, their inability to retain appreciable amounts of nitrogen from the ingested urea, as shown by the nitrogen

balances, may be taken as evidence of their nearly if not quite complete inability to utilize the urea nitrogen for the formation of protein.

It is not possible, of course, to determine from the balances of nitrogen whether an exchange took place between the nitrogen of the dietary urea and that of tissue proteins comparable to the exchange of nitrogen between dietary ammonia and tissue proteins as observed by Rittenberg and his co-workers ('39).

TABLE 3

*Hourly heat production of rats per 200 gm. of empty body weight and total respiratory quotients as influenced by the addition of 2.0 gm. of urea to a basal maintenance ration*

| | RAT NO. | | | | | | AVERAGE |
|---|---|---|---|---|---|---|---|
| | 31 | 32 | 33 | 34 | 35 | 36 | |
| Basal ration, 8.0 gm. daily | | | | | | | |
| Initial period | | | | | | | |
| Heat production, calories per hour | 913 | 929 | 925 | 870 | 886 | 979 | 917 |
| Respiratory quotient | 0.90 | 0.90 | 0.91 | 0.92 | 0.93 | 0.90 | 0.91 |
| Final period | | | | | | | |
| Heat production, calories per hour | 872 | 872 | 815 | 808 | 825 | 962 | 859 |
| Respiratory quotient | 0.91 | 0.92 | 0.95 | 0.95 | 0.93 | 0.93 | 0.93 |
| Average of initial and final periods | | | | | | | |
| Heat production, calories per hour | 893 | 901 | 870 | 839 | 856 | 971 | 888 |
| Basal ration plus 2.0 gm. of urea | | | | | | | |
| Heat production, calories per hour | 863 | 858 | 896 | 917 | 855 | 990 | 896 |
| Respiratory quotient | 0.90 | 0.91 | 0.90 | 0.92 | 0.93 | 0.91 | 0.91 |
| Increase in heat production due to uren, calories per hour | −30 | −43 | +26 | +78 | −1 | +19 | +8 |

Table 3 presents the data for the average hourly heat production of the individual rats computed per 200 gm. empty body weight, and the total respiratory quotients. The individual hourly measurements of $CO_2$ production, except those of the first hour of observation, are characterized as being very uniform; and of these, it was found necessary to omit from the averages only three single hourly determinations as being significantly affected by activity.

The effect of the feeding of urea on the heat production is shown by the comparison of the heat production representing

the urea-supplemented ration with the average heat production of the initial and final basal periods. Only one animal (rat 34) shows an appreciable increase in heat production as a result of the addition of urea to the basal ration. With the remaining five rats the differences in heat production attributable to urea are small, and they include some negative values. The average increase resulting from the feeding of urea is 8 gm.-calories per hour. This represents an increase of less than 1% of the heat production representing the basal ration and is not significant.

The respiratory quotients were not significantly altered by the addition of urea to the basal ration.

The foregoing observations lead to the conclusion that the feeding of urea, and its consequent excretion through the kidneys, did not appreciably affect the energy metabolism of the rats.

It appears that in rats, in contrast to ruminants and possibly swine, the synthesis of protein from urea does not occur in the intestinal tract to any significant extent.

## SUMMARY AND CONCLUSIONS

Urea fed to rats as a supplement to a mixed maintenance ration, in quantities of 2 gm. per day, was almost entirely recovered as such in the urine and feces, as indicated by the balances of nitrogen, carbon and energy and by the ratios of carbon to nitrogen and energy to nitrogen in the urine. Of the total nitrogen ingested as urea 95.5% was recovered in the urine and 0.7% in the feces. Of the carbon and energy of the urea fed 98.5% and 99.8%, respectively, were found in the urine.

The feeding of urea showed no significant effects on either the heat production or the respiratory quotients.

The data lead to the conclusions that rats do not utilize urea to any significant extent, and that the excretion of urea does not exert a specific dynamic effect.

## LITERATURE CITED


BARTLETT, S., AND A. G. COTTON 1938 Urea as a protein substitute in the diet of young cattle. J. Dairy Research, vol. 9, p. 263.

BORSOOK, H., AND H. M. WINEGARDEN 1931 a The work of the kidney in the production of urine. Proc. Nat. Acad. Sci., vol. 17, p. 3.

———— 1931 b The energy cost of the excretion of urine. Proc. Nat. Acad. Sci., vol. 17, p. 13.

———— 1931 c On the specific dynamic action of protein. Proc. Nat. Acad. Sci., vol. 17, p. 75.

CARPENTER, T. M. 1938 The effect of urea on the human respiratory exchange and alveolar carbon dioxide. J. Nutrition, vol. 15, p. 499.

EATON, A. G., S. C. CORDILL AND J. L. GOUAUX 1935 Is the work of the kidney, due to the excretion of urea, a factor in specific dynamic action? Am. J. Physiol., vol. 113, p. 37. J. Nutrition, vol. 10, p. 429.

FINGERLING, G., B. HIENTSCH, R. KUNZE AND K. REIFGERST 1937 Ersatz des Nahrungseiweisses durch Harnstoff beim wachsenden Rinde. Bd. 128, S. 221.

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, p. 535.

GRAFE, E. 1915 Beitrage zur Kenntnis der Ursachen der spezifisch-dynamischen Wirkung der Eiweisskörper Deutsch. Arch. f. Klin. Med., Bd. 118, S. 1.

HART, E. B., G. BOHSTEDT, H. J. DEOBALD AND M. I. WEGNER 1938 The utilization of the nitrogen of urea and ammonium bicarbonate by growing calves. Proc. Am. Soc. Animal Production, vol. 31, p. 333–336.

HONCAMP, F., AND ST. KOUDELA 1927 Untersuchungen über die Verwertung von Ammoniaksalzen und von Harnstoff als Eiweissersatz für die Lebenderhaltung und den Fleischansatz, sowie für die Milchbildung beim landwirtschaftlichen Nutzvieh. Zeitschr. Tierzucht und Züchtungsbiol., Bd. 10, S. 1–46.

KOCHER, R. A., AND H. C. TORBERT 1932 The effect of urea ingestion on the nitrogen partition of the urine at endogenous nitrogen level. J. Biol. Chem., vol. 95, p. 427.

KREBS, K. 1937 Der Wert der Amide bei der Fütterung des Rindes. Biedermanns Zentr. B. Tierernähr., Bd. 9, S. 394.

KRISS, MAX 1938 The specific dynamic effects of proteins when added in different amounts to a maintenance ration. J. Nutrition, vol. 15, p. 565.

KRISS, MAX, AND LEROY VORIS 1937 A further contribution to the derivation of factors for computing the gaseous exchange and heat production in the metabolism of protein. J. Nutrition, vol. 14, p. 215.

LUBLIN, A. 1928 Über den Einfluss der Nierenarbeit auf den Gaswechsel des Menschen. Zeit. f. klin. Med., Bd. 109, S. 371.

LUSK, G. 1912 Animal calorimetry. III. Metabolism after the ingestion of dextrose and fat, including the behavior of water, urea and sodium chloride solutions. J. Biol. Chem., vol. 13, p. 27.

MOORE, D. D., P. H. LAVIETES, A. M. WAKEMAN AND J. P. PETERS 1931 The effect of ingested urea on nitrogen metabolism. J. Biol. Chem., vol. 91, p. 373.

PIEPENBROCK, A. 1927 The problem of the substitution of urea for protein in animal nutrition. Fortschritte Landw., Bd. 2, S. 650–656.

RAJZMAN, A. 1936 Action dynamique spécifique et activité rénale. Arch. internat. Physiol., vol. 43, p. 423.

RITTENBERG, D., R. SCHOENHEIMER AND A. S. KESTON 1939 The utilization of ammonia by normal rats on a stock diet. J. Biol. Chem., vol. 128, p. 603.

SAUER, F. 1938 The role of urea in the metabolism of ruminants. Biedermanns Zentr. B. Tierernähr., Bd. 10, S. 213.

SCHMIDT, J., J. KLIESCH, A. KAMPFFER AND K. KREBS 1937 Experiments with urea and glycine as protein substitutes in the feeding of milk cows. Forschungsdienst., Bd. 4, S. 229–243.

TANGL, F. 1911 Die Arbeit der Nieren und die "spezifischdynamische Wirkung" der Nährstoffe. Biochem. Zeit., Bd. 34, S. 1.

TAYLOR, A. E., AND A. I. RINGER 1913 The utilization of ammonia in the protein metabolism. J. Biol. Chem., vol. 14, p. 407.

# PASTURE STUDIES XVI

# THE NUTRITIVE VALUES OF KENTUCKY BLUE GRASS, RED TOP AND BROME GRASS

## WITH PARTICULAR REFERENCE TO THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF THE HERBAGES AND THE LIVE WEIGHT GAINS MADE BY THE ANIMALS SUBSISTING THEREON [1]

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Our present information from biological tests of the relative feeding values of the several species of grasses and clovers commonly occurring in mixed pasture herbage, is decidedly meager. It seems certain, however, that the herbage from different species of plants has an intrinsic nutritive value characteristic for each species (Knott, Hodgson and Ellington, '34; Kirsch, '33; Pollard and Chibnall, '34; Garrigus and Rusk, '35; Crampton, '34; Crampton and Cameron, '36).

Tests at Macdonald College (Crampton and Forshaw, '39) with rabbits have also indicated a definite intra-seasonal trend in the feeding value of mixed pasture herbage. Clippings of herbage grown during spring and fall supported normal growth of rabbits, while material grown during midsummer under less favorable climatic conditions proved of

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decidedly poor value. In these studies interpretation of the results was complicated by the change in botanical composition of the herbage with advancing season. It has been shown (Dore, '36) that, in the permanent pastures of Quebec province, wide variations may exist between the proportions of the various species in a pasture as a result of management and of climatic conditions. To eliminate this factor it became apparent that clippings from pure stands of the grasses to be studied would be necessary and in 1938 such material was made available.

Furthermore, it was becoming increasingly evident that differences in nutritive value of pasturage as measured by the live weight gains of rabbits were frequently not predictable from the usual chemical feeding stuffs analysis (Crampton, '39). This situation, if applicable also to the nutritive value of such materials for larger animals, is disturbing in view of the large amount of pasture research already completed in which chemical analyses alone are the criterion of 'nutritive value.'

The literature contains strong evidence (Crampton, '39) that the usefulness of the partition of the carbohydrates into crude fiber and nitrogen-free extract for the purpose of describing nutritive value is to be seriously questioned. Norman ('39) states, "It would be an important advance if the determination of the crude fibre fraction and its use in expressing composition were abandoned as inadequate, unreliable, and misleading." He further states, "The most serious criticism of the present system of analyses used for grasses and forage crops is that there is included no measure of the lignin content."

It seemed desirable, therefore, in the 1938 feeding trials to design the experiment in such a way as to permit a critical statistical examination of the relation between the chemical composition (proximate principles of the feeding stuffs analysis) of the clippings fed and the growth of the animals subsisting thereon.

## EXPERIMENTAL PART

### *Diets*

From large plots (¼ to ½ acres) of pure stands of brome, Kentucky blue and red top grass clippings were made at about 16-day intervals throughout the summer of 1938. These clippings were dried in a forced draught hot air dryer and subsequently ground in a hammer mill to pass a $^{15}/_{32}$ inch screen. In order to obtain sufficient herbage for the feeding periods it was necessary with all three grasses to combine the clippings of July 11th and August 4th and those of August 29th and September 19th. Thus at the end of the growing season five samples of herbage clippings were available from each of the three grasses above mentioned. The chemical analyses of these fifteen lots of herbage are given in table 1.

### *Animals*

Sixty young growing rabbits, averaging 1500 gm., were allotted equally to fifteen groups, each group to be fed on a different clipping.

All animals were penned and fed individually. The feeding practice and equipment have been previously described (Crampton, '34). The trials were of 35 days duration. The first 7 days on feed were treated as a preliminary period while the last 28 days constituted the test period proper. Throughout the trial feed was provided ad libitum. Weekly live weight gain and feed intake data were recorded for each animal.

## STATISTICAL ANALYSIS OF DATA

The method of partial regression was employed as suggested by Crampton and Hopkins ('34) to adjust the observed live weight gains of the rabbits in order to remove the effects on gain of varying levels of feed intake and of different initial weights of rabbits. The initial weights shown in table 2 are those at the beginning of the test periods, and are those involved in the adjustment of gains above referred to.

In an attempt to evaluate the usefulness of chemical analyses as an index of nutritive value of pasture herbage simple, partial and multiple correlations were calculated between the average 28-day gains of the rabbits of each lot and the feed fractions isolated (1) by the standard scheme of feeding

TABLE 1

*Chemical analysis of dry matter of clippings used*

| DATE | SPECIES | NITROGEN[1] | ETHER EXTRACT[1] | ASH[1] | CRUDE FIBER[1] | N-FREE EXTRACT[2] | LIGNIN[3] | CELLULOSE[4] | OTHER $CH_2O$[5] |
|---|---|---|---|---|---|---|---|---|---|
| May 26 | Brome | 4.3 | 2.6 | 15.9 | 15.6 | 39.3 | 6.8 | 19.2 | 28.9 |
| | Kentucky blue | 4.0 | 2.5 | 11.5 | 21.7 | 39.5 | 5.3 | 20.6 | 35.3 |
| | Red top | 3.6 | 3.1 | 11.8 | 18.7 | 40.1 | 5.8 | 19.2 | 33.8 |
| June 10 | Brome | 4.0 | 3.2 | 16.9 | 19.1 | 35.6 | 7.3 | 20.9 | 26.5 |
| | Kentucky blue | 3.3 | 2.7 | 11.8 | 22.4 | 42.8 | 7.3 | 23.0 | 34.9 |
| | Red top | 3.1 | 2.7 | 11.2 | 23.7 | 59.4 | 7.2 | 24.4 | 51.5 |
| June 23 | Brome | 3.4 | 4.6 | 14.0 | 23.0 | 37.3 | 7.8 | 22.0 | 31.1 |
| | Kentucky blue | 3.7 | 3.9 | 12.0 | 23.9 | 37.3 | 8.0 | 25.5 | 27.7 |
| | Red top | 3.3 | 3.9 | 11.8 | 26.8 | 36.9 | 8.7 | 26.8 | 28.2 |
| July 11 – Aug. 4 | Brome | 3.5 | 4.0 | 11.9 | 21.0 | 41.0 | 6.8 | 22.6 | 32.6 |
| | Kentucky blue | 2.6 | 3.5 | 10.4 | 23.5 | 46.2 | 9.3 | 22.8 | 37.6 |
| | Red top | 3.1 | 3.4 | 9.9 | 24.7 | 42.4 | 10.5 | 25.0 | 31.6 |
| Aug. 29 – Sept. 19 | Brome | 3.8 | 2.8 | 13.5 | 17.9 | 42.4 | 7.6 | 19.1 | 33.6 |
| | Kentucky blue | 4.1 | 2.7 | 12.2 | 18.7 | 41.0 | 9.8 | 19.1 | 30.8 |
| | Red top | 3.8 | 2.6 | 13.1 | 18.0 | 42.7 | 9.1 | 18.7 | 32.9 |

[1] Official A. O. A. C. methods.

[2] N-free extract = D.M.—((nitrogen × 6.25) + ether extract + total ash + crude fiber).

[3] Determined by method proposed by Crampton and Maynard ('38) with the modification that acid hardened filter paper replaced bolting silk in the recovery of the pepsin digestion residue and that the boiling of the chloroform-acetic acid suspension of lignin was discontinued immediately the surface scum broke, and that diatamaceous earth in place of asbestos was used in the final lignin filtration.

[4] Determined by method proposed by Crampton and Maynard ('38).

[5] Other carbohydrates = D.M. —((Nitrogen × 6.25) + ether extract + totol ash + lignin + cellulose).

stuffs analysis and (2) by the scheme of analysis proposed by Crampton and Maynard ('38). In addition standard and partial regression coefficients [4] were determined using in each instance the 28-day gains as the dependent, and the feed fractions (as protein, fiber, etc.) as independent variables.

[4] Snedecor—Statistical Methods, 1937, Chapter 13.

TABLE 2

*Initial weights of rabbits, dry matter consumption and live weight gain data*

| DATE | SPECIES | INITIAL WEIGHT | DRY MATTER CONSUMPTION | 28-DAY GAINS | 28-DAY GAINS ADJUSTED FOR DEVIATIONS FROM THE GENERAL MEANS OF INITIAL WEIGHT AND DRY MATTER CONSUMPTION |
|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | |
| | Brome | 1911 | 2905 | 261.25 | 280 |
| May 26 | Kentucky blue | 1889 | 3150 | 253 | 269 |
| | Red top | 2006 | 3397 | 274.5 | 187 |
| | Average | 1935 | 3067 | 262.92 | |
| | Brome | 1505 | 2524 | 85.75 | 128 |
| June 10 | Kentucky blue | 1586 | 2910 | 170.5 | 131 |
| | Red top | 1553 | 3059 | 221.75 | 139 |
| | Average | 1548 | 2831 | 159.33 | |
| | Brome | 1383 | 2635 | 109.75 | 103 |
| June 23 | Kentucky blue | 1300 | 2692 | 38.75 | 3 |
| | Red top | 1335 | 2507 | —110.25 | —94 |
| | Average | 1339 | 2612 | 12.75 | |
| July 11 to Aug. 4 | Brome | 1398 | 2473 | 147.5 | 184 |
| | Kentucky blue | 1365 | 2353 | —149.0 | —89 |
| | Red top | 1384 | 2741 | 104 | 71 |
| | Average | 1382 | 2522 | 34.17 | |
| Aug. 29 to Sept. 19 | Brome | 1382 | 2621 | 259.75 | 256 |
| | Kentucky blue | 1361 | 2314 | 83.25 | 152 |
| | Red top | 1314 | 2428 | 178.75 | 211 |
| | Average | 1352 | 2454 | 173.92 | |
| Lot averages | Brome | 1516 | 2631 | 172.8 | 190 |
| | Kentucky blue | 1500 | 2634 | 79.3 | 93 |
| | Red top | 1518 | 2826 | 133.75 | 103 |
| General average | | 1511 | 2697 | 128.62 | 129 |

## RESULTS AND DISCUSSION

The 28-day gains, adjusted for differences in initial weight and feed intake, are given by clippings and by species of grass in table 3.

A perusal of these gains shows that in addition to intra-seasonal variations in growth promoting ability there are significant differences between species. Also there appear to

be differences in the part of the season at which these grasses display signs of declining nutritive value. While all three species show a mid-season falling off in nutritive value the 'low' is not as marked with brome grass as in the case of either Kentucky blue or red top.

Particularly poor growth was obtained with those clippings of Kentucky blue grass and red top taken during the mid-summer. In fact two rabbits on the Kentucky blue grass and one on the red top of July 11th and August 4th clipping

TABLE 3

*Twenty-eight-day gains of rabbits adjusted for differences in initial weight and feed intake*

| DATE OF CLIPPING | BROME GRASS | KENTUCKY BLUE | RED TOP | CLIPPING MEAN |
|---|---|---|---|---|
| | *gm.* | *gm.* | *gm.* | *gm.* |
| May 26 | 280 | 269 | 187 | 245 |
| June 10 | 128 | 131 | 135 | 132 |
| June 23 | 103 | 3 | —94 | 4 |
| July 11 } Aug. 4 } | 184 | —89 | 71 | 55 |
| Aug. 19 } Sept. 19 } | 256 | 152 | 211 | 207 |
| Species average | 190 | 93 | 103 | 129 |

Necessary difference (with P = 0.05) between (1) means of clippings of same species = 98 gm.; (2) means of species with same date of clipping = 98 gm.
Necessary difference between clipping means = 56 gm.
Necessary difference between species means = 44 gm.

died before the completion of the 35-day feeding period. The data for these rabbits were deleted and three replacements used to complete the data for the test. While these replacements succeeded in living through the feeding period their growth response paralleled those of the others of the group.

At the completion of the 35-day period, one of the replacement rabbits on Kentucky blue grass and one of the original animals that survived the first 28-day test feeding period were continued, the latter (no. 1) for 28 days and the former (no. 2)

for 9 days on the same diet with the addition of 10 gm. daily of cornstarch. The results are shown below.

| RABBIT | AVERAGE DAILY GAIN ON KENTUCKY BLUE GRASS GROWN BETWEEN JUNE 23RD AND AUGUST 4TH | AVERAGE DAILY GAIN ON SAME GRASS PLUS 10 GM. DAILY OF CORNSTARCH |
|---|---|---|
| | *gm.* | *gm.* |
| 1 | −4.5 | +9.09 |
| 2 | +1.0 | +4.4 |

The results secured by supplementing the diets with cornstarch and the condition of the animals at the completion of the trial would indicate that lack of available energy was at least one of the factors responsible for the decline in growth noted with mid-summer herbage. This is in agreement with the findings of Crampton and Finlayson ('35) and Crampton and Cameron ('36) using casein and cane sugar as supplements respectively.

The results of the statistical studies are summarized in table 4.

*Simple and partial correlations*

Before proceeding further, attention is called to the magnitude of the correlations needed for statistical significance shown in table 4 a. The fact that they are large numerically is due to the small number of degrees of freedom available for the usual tests of significance. It may be pointed out that significance is probability. Thus when a correlation may not in nineteen out of twenty cases prove real (i.e., significant with odds of 19:1 or $P = 0.05$) it may prove real in eighteen cases out of twenty (i.e., $P = 0.10$), etc. Thus the correlation obtained is the best estimate of the facts from the data. Our dependence on them is determined by what arbitrary limit of odds we demand, which after all is in part a matter of judgment of the case.

In this study it was necessary in calculating the correlations to treat the average gains of the four rabbits on each diet as single observations. Thus the quantities correlated were not individual observations but means of four, which

will be correspondingly less subject to the irregular fluctuations obscuring true correlations. The reduction in the significance of the correlations obtained is therefore probably

TABLE 4

*Correlations between 28-day gains of rabbits and fractions isolated by feeding stuffs analyses*

| PROXIMATE PRINCIPLE | SIMPLE CORRELATION COEFFICIENTS (r) | PARTIAL CORRELATION | STANDARD REGRESSION COEFFICIENTS | | PARTIAL REGRESSION COEFFICIENTS (b) | MULTIPLE CORRELATIONS |
|---|---|---|---|---|---|---|
| | | | Actual | Relative | | |
| | | | | % | | |
| **Standard analysis** | | | | | | |
| Crude protein | +0.6582 | +0.7274 | +0.6933 | 35.6 | +28.61 | |
| Ether extract | −0.7815 | −0.5944 | −0.4394 | 22.6 | −75.33 | $R^2 = 0.863$ |
| Ash | +0.1864 | −0.6546 | −0.4272 | 22.0 | −35.63 | |
| Crude fiber | −0.7436 | −0.2002 | −0.1383 | 7.1 | − 5.32 | $R = 0.929$ |
| N-free extract | +0.1619 | +0.4774 | +0.2468 | 12.7 | + 5.06 | |
| **Modified scheme** [1] | | | | | | |
| Crude protein | +0.6582 | +0.6950 | +0.5757 | 29.7 | +23.73 | |
| Ether extract | −0.7815 | −0.5556 | −0.3281 | 16.9 | −56.40 | $R^2 = 0.905$ |
| Ash | +0.1864 | −0.6864 | −0.3944 | 20.4 | −32.89 | |
| Lignin | −0.4866 | −0.5413 | −0.1857 | 9.6 | −14.42 | $R = 0.952$ |
| Cellulose | −0.7396 | −0.5501 | −0.2935 | 15.3 | −12.58 | |
| Other carbohydate | +0.2266 | +0.3014 | +0.1596 | 8.2 | + 3.09 | |

[1] Crampton and Maynard ('38).

TABLE 4a

*Correlation coefficients necessary for statistical significance ($P = 0.05$)*

| | DEGREES OF FREEDOM | STANDARD ANALYSIS | MODIFIED ANALYSIS |
|---|---|---|---|
| Simple correlations | 13 | 0.532 | 0.532 |
| Partial correlations | | | |
| Standard analysis | 7 | 0.666 | |
| Modified scheme | 6 | | 0.707 |
| Multiple correlations | 7 and 6 | 0.860 | |
| | 6 and 7 | | 0.900 |

more apparent than real. The interpretation of these statistics in the discussion following has been made with the peculiarities and limitations of these data in mind.

Simple correlations in data of this type must be interpreted in the sense of cause and effect with extreme caution, for obviously there is not likely to be a direct causal relationship between any of the feed fractions and the live weight gain of the rabbits. For example, the crude fiber correlation of —0.7436 doubtless results from the common association of crude protein with both gain and fiber.

Some of the difficulty from common associations may be eliminated by using partial rather than simple correlations. When this method is applied it is seen that much of the apparent relation of crude fiber to gains has been eliminated; that of crude protein and of nitrogen-free extract has been increased. Ash also becomes of importance. This is in accord with the diluent effect to be expected of any inorganic substance present to the extent of 10 to 17% of the total dry matter of the diet (see table 1).

From the squared multiple correlation coefficient ($R^2$) it is seen that in the case of the standard analysis scheme 86.3% of the variation in gains is accounted for by the five feed fractions isolated; and in the case of the modified plan, 90.5% is the result of the six fractions considered.

From the standard regression coefficients one may obtain an idea of the relative importance of any one of the proximate principles in causing variability in gain. Thus of the 86.3% of variability in gain accounted for by the five fractions of the standard feed analysis, some 35.6% is traceable to variations in protein content, 22.6% to ether extract, etc.

It appears from these figures that crude fiber and nitrogen-free extract have probably played minor roles in this connection as compared to protein, fat and ash. In the modified plan of analysis, all values are reduced due to the inclusion of six instead of five fractions. Nevertheless, in this latter plan, the total weight of the three carbohydrate fractions is 33.1% of the variability accounted for by the analysis as compared to 19.8% in the standard analysis; from which it might be argued that some improvement in the chemical

description of the growth promoting properties of the feed has been effected by the modified plan.

Partial regression coefficients indicate the change in gain (grams live weight in 28 days) to be expected with a change of one unit of per cent in the feed fraction in question.

The effect of the fractions other than those of the carbohydrate group may at first appear large. It will be noted, however, that changes in chemical composition in these fractions are not large in relation to the marked differences in the gains obtained on these several clippings. Furthermore it is by no means probable, for example, that an increase of 1% of ether extract is nutritionally merely an increase in a high energy utilizable food material.

Digestion studies during 1938 at Macdonald College (unpublished) on diets of pasture herbage both with steers and with rabbits show ether extract to be variable in digestibility. For example, in pasture herbage grown during mid-summer the ether extract content is greater than that found either in spring or fall grown herbage. The digestibility of this ether extract fraction of the summer herbage is, however, markedly lower. The increase in ether extract is probably due to the presence of seeds in the mature summer herbage. These seeds, however, largely escape digestion, thus increasing the ether extract of the feces. In green herbage the fact that ether removes such substances as chlorophyl, phytosterol and phosphatides, as well as the triglycerides and free fatty acids, also contributes to the variable utilization by animals which this feed fraction may have. Furthermore an increase in ether extract may be accompanied by qualitative changes in other fractions which result in lowered feeding value. Thus, change in the ether extract content may be an index of change in feeding value much greater than a simple addition effect of the ether extract itself. This is doubtless in part the situation with ash though here the diluent effect is probably the greater. It is likely that the rather high ash values indicate the incidental but unavoidable inclusion of some soil with the lawn mower clippings.

Protein being highly digested was related to gains in a positive way. In view of the results with cornstarch already mentioned, it seemed improbable that this correlation meant that even the lowest protein content (16.25%) represented suboptimum protein intake. Rather, due to the unavailability of carbohydrate and fats, the protein was providing an important source of energy; and increases in protein meant increases in available dietary energy and hence increases in live weight gains.

Admittedly the problem is by no means solved. It is evident that the present scheme of feeding stuffs analysis is quite inadequate for describing the growth promoting properties of the energy yielding nutrients of pasture herbage. Hence, the assumption that samples of pasture herbage are of different feeding value because of differences in chemical composition cannot be accepted as warranted.

## SUMMARY AND CONCLUSIONS

Evidence is presented that in addition to differences in the nutritive value of different species of grasses commonly found in the pastures of Eastern Canada, the feeding value of the herbage of any single species changes during the growing season. Herbage grown during spring and fall, when plant growing conditions are favorable, is of excellent feeding value. On the contrary, herbage grown during mid-summer is of lower nutritive value, due apparently to a reduction in the availability to the animal of the carbohydrate fractions.

Statistical examination of the data indicates that simple correlations between growth of the animals and the fractions of the usual feeding stuffs analysis may frequently point to erroneous conclusions when interpreted in the sense of cause and effect. Partial correlation changes not only the magnitude but also the sign of some of the coefficients. In particular it may be noted that variations in crude fiber, which under simple correlations appear important, probably actually have only minor effects on live weight gains. Also ether extract and ash are highly negatively correlated with gain.

Protein content is highly correlated with gain in weight of animal. However, there is no evidence that protein as such is a limiting factor in the growth promoting value of immature pasture herbage, but rather that increases in protein probably represent merely increases in available energy.

## LITERATURE CITED


CRAMPTON, E. W. 1934 Pasture studies IV. The nutritive value of pasture herbage—quality of protein. Emp. J. Exp. Agr., vol. 2, p. 337.

———— 1939 Pasture studies XIV. The nutritive value of pasture herbage. Some problems in its estimation and some results thus far obtained at Macdonald College. Scientific Agriculture, vol. 19, p. 345.

CRAMPTON, E. W., AND C. D. T. CAMERON 1936 The nutritive value of pasture herbage. Unpublished data. Macdonald College.

CRAMPTON, E. W., AND D. A. FINLAYSON 1935 Pasture studies VII. The effect of fertilization on the nutritive value of pasture grass. Emp. J. Exp. Agr., vol. 3, p. 331.

CRAMPTON, E. W., AND R. P. FORSHAW 1939 Pasture studies XV. The intra-seasonal changes in the nutritive value of pasture grass. Scientific Agriculture, vol. 19, p. 701.

CRAMPTON, E. W., AND J. W. HOPKINS 1934 The use of the method of partial regression in the analysis of comparative feeding trial data. II. J. Nutrition, vol. 8, p. 329.

CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition, vol. 15, p. 383.

DORE, W. G. 1936 Pasture studies X. Succession and variation in the botanical composition of permanent pastures. Scientific Agriculture, vol. 16, p. 569.

GARRIGUS, W. P., AND H. P. RUSK 1935 Record of proceedings of the twenty-eighth annual meeting of the American Society of Animal Production, p. 75.

KIRSCH, W. 1933 Recent research on forage crop cultivation, conservation and utilization at the Animal Breeding Institute of the University, Königsberg. Imp. Bureau Plant Genetics, Bul. no. 8.

KNOTT, J. C., R. E. HODGSON AND E. V. ELLINGTON 1934 Nutritive value of reed canary grass vs. wheat pasture. Wash. Agr. Exp. Sta. Bul. 295.

NORMAN, A. C. 1939 Biochemical approach to grass problems. J. Am. Soc. of Agronomy, vol. 31, p. 751.

POLLARD, A., AND A. C. CHIBNALL 1934 Proteins of grasses III. Biochem. J. vol. 28, p. 326.

# THE RECTAL TEMPERATURE AND THE METABOLISM OF THE WILD COTTONTAIL RABBIT

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To contribute information as to whether there are metabolic differences between animals in the wild and the domestic state, measurements were made on eight wild cottontail rabbits (Lepus sylvaticus). These were snared in Oklahoma on November 29, 1937, and were either mature or nearly so. Measurements of rectal temperature and metabolism were made by the methods previously used for domestic rabbits (Lee, '39 a, b).

*Rectal temperature.* As there is a temperature gradient in the rectum of the wild rabbit, a condition also found in the domestic rabbit, the thermo-junction should be inserted 150 mm. to obtain valid body temperature measurements. The gradient between the temperatures at 75- and 150-mm. depths averaged 0.3°C. at an air temperature of 28°C. and was slightly less at lower air temperatures. Fasting enforced for 24 hours did not alter the rectal temperature. Between environmental temperatures of 16° and 28° the body temperature remained constant, but when the rabbits had been 24 hours at 10° to 12°C., it was lowered 0.7°C., on the average. Three to 4 minutes of activity caused increases in the temperature of 0.3° to 2.1°C., the maximum being greater than that noted with our domestic rabbits after similar exercise and approaching that of 2.5° reported by Purdy ('25). At environmental temperatures of 15° to 28°C., when the rabbits had food available or when fasting had been enforced up

to 24 hours, the range in rectal temperatures (eighty-four measurements) at a depth of 150 mm. was from 38.7° to 40.9°C. The average was 39.9°C., nearly the same as that of our domestic rabbits (39.6°). There was no sex difference.

*Prerequisites for basal metabolism measurements.* The wild rabbits were quiet, as a rule, but any periods of activity were rejected and only the values obtained in quiet periods and agreeing within 10% of the minimum measurement on each day were used in calculating the average metabolism. The zone of thermic neutrality was found to be between 28° and 30°C. Measurements at 28° and then at 30°C. on the same day gave the following results, expressed as calories per square meter of surface area ($S = \frac{K \times w^{2/3}}{10,000}$ or $S = 0.001w^{2/3}$) per 24 hours:

| Rabbit no. | 11 | 12 | 13 | 15 | 16 | 19 | Average |
|---|---|---|---|---|---|---|---|
| 28°C. | 561 | 631 | 713 | 611 | 659 | 717 | 649 |
| 30°C. | 539 | 671 | 700 | 598 | 661 | 712 | 647 |

These measurements were made after the rabbits (fasting 24 hours) had been living previously at a moderate temperature and during the 24 hours just beforehand at 28°C. Observations above 30° and below 28° gave higher values. A series at 28° immediately after the rabbits had been removed from an environment of 12°C. gave results from 6 to 29% above subsequent measurements after 24 hours at 28°C. After 22 to 30 hours of enforced fasting at 28° to 30°C. (exact length of fasting unknown, because of uncertainty as to when the rabbits ate last), the respiratory quotients ranged from 0.69 to 0.82 and averaged 0.73. The heat production reached a fairly uniform level after 24 hours of fasting at thermic neutrality and did not decrease when the fast was continued for several hours longer. The prerequisites for basal metabolism measurements on the wild rabbit are, therefore, absence of activity, thermic neutrality, 24 hours of habituation to thermic neutrality, and fasting for 24 hours at this temperature.

*Basal metabolism.* The basal metabolism of each of the rabbits was determined at intervals over 8 months (table 1). With five of the eight rabbits the heat production per square meter

of body surface was highest in the initial experiment. For rabbits 19, 20, and 22 the environmental temperature previous to their initial experiments was only 18°C., which was undoubtedly a contributory cause of their first extraordinarily high measurements. In the second series of experiments, about 3 weeks

TABLE 1

*Basal metabolism of the wild rabbit*

| Rabbit no. and sex | 11 ♀ | 12 ♂ | 13 ♂ | 15 ♂ | 16 ♂ | 19 ♀ | 20 ♀ | 22 ♀ |
|---|---|---|---|---|---|---|---|---|
| Average weight, kg. | 0.97 | 0.90 | 0.96 | 0.87 | 0.90 | 1.01 | 1.02 | 1.22 |
| APPROXIMATE DATE | CALORIES PER SQUARE METER [1] PER 24 HOURS | | | | | | | |
| December 5 [2] | 628 | 799 | 780 | 700 | 753 | 816 [3] | 906 [3] | 791 [3] |
| December 27 | 649 | 704 | 804 | 751 | 659 | 726 | 667 | 722 |
| February 15 | 573 | 666 | 730 | 729 | 653 | | 624 | 644 |
| February 25 | | | | | | 672 | | 615 |
| March 2 | 606 | | | | | | | 584 |
| March 15 | 622 | | | 672 | | | 657 | 576 |
| March 20 | 570 | 656 | | 764 | | 713 | 648 | 526 |
| March 25 | | 726 | 634 | 648 | 619 | 699 | 624 | 571 |
| April 4 | 561 | 631 | 713 | 611 | 659 | 717 | 592 | 558 |
| May 10 | 557 | 665 | 732 | 671 | 643 | 602 | 531 | 573 |
| July 15 | | 626 | 710 | 649 | | | | |
| Average [4] | 591 | 668 | 721 | 687 | 647 | 688 | 620 | 597 |
| | TOTAL HEAT PRODUCTION PER 24 HOURS | | | | | | | |
| Average | 59.2 | 62.7 | 70.8 | 62.3 | 61.5 | 70.4 | 63.7 | 68.8 |

Measured at 28° to 30°C., after 22 to 30 hours of enforced fasting; habituated to experimental temperature for 24 hours prior to experiment.

[1] Weight in grams raised to the two-thirds power and multiplied by 0.001.

[2] First experiment.

[3] Rabbits 19, 20 and 22 were not at 28° to 30° prior to first experiment but at 18°C.

[4] Excluding first experiment.

later, most of the rabbits had a higher metabolism than in succeeding measurements. Probably prior to their arrival at the laboratory (December 2, 1937) they had been living at much lower temperatures than usually prevailed at the laboratory, and adjustment to moderate temperatures had not fully taken place even at the time of the second measurements. Any decrease in metabolism as the animals' stay in the laboratory be-

came longer may have been the result of habituation to a higher and narrower range of environmental temperatures rather than of adaptation to handling. As the metabolism is predisposed to be high in the first experiment with any animal, the first experiment has been omitted from the general average in each case.

The measurements on the individual animals were fairly uniform. The maximum variation from the individual averages was +21% with rabbit 22 and ±14% or less with the others. The standard deviation of the percentage differences of the daily average values from the general average for each rabbit was ±6.7%, comparable to that of ±6.9% noted with our domestic rabbits. The average heat production of the individual wild rabbits ranged from 591 to 721 calories per square meter, and the general average for the eight rabbits was 652 calories.

The average total 24-hour heat production ranged from 59 to 71 calories and averaged 64.9 calories (average weight, 0.98 kg.). This is 6% above the average total heat production of our domestic rabbits of like weight (Lee, '39c) and recalls the finding of Benedict and Petřík ('30) that wild rats have a higher metabolism than laboratory-bred albino rats.

There was no consistent relationship between variations in rectal temperature and variations in basal metabolism.

The heat production of the four females averaged 8% below that of the four males. This sex difference is greater than the 2% difference found with our domestic rabbits. It is questionable, however, whether this finding (8%) based on so few values is any more than suggestive of the trend.

*Reaction of metabolism to environmental temperature.* The fasting heat production (calories per square meter) of three wild rabbits measured at 16° after at least 24 hours' habituation to this temperature and their average basal metabolism at 28°C. were as follows:

| Temp. | No. 19 | No. 20 | No. 22 |
|---|---|---|---|
| 16°C. | 930 | 1011 | 901 |
| 28°C. | 688 | 620 | 597 |

At 16°, with no indication of shivering shown by the activity records, the heat production was increased, on the average, 49% above the average basal metabolism at 28°C.

## SUMMARY

The average rectal temperature of eight wild cottontail rabbits, essentially adult and having an average weight of 0.98 kg., was 39.9°C. at a depth of 150 mm. in the rectum. The temperature increased as the depth in the rectum at which the measurement was made increased. Between the 75- and 150-mm. depths, the average difference in temperature was 0.3°C. (environmental temperature, 28°). Fasting enforced for 24 hours, at air temperatures between 10° and 28°, did not alter the body temperature. Environmental temperatures between 16° and 28° had no effect, but at 10° to 12°C. the average rectal temperature was lowered 0.7°. Three to 4 minutes of exercise caused increases in temperature of 0.3° to 2.1°C.

The average total heat production per 24 hours was 64.9 calories, and the average heat production per square meter of surface area ($S = 0.001w^{2/3}$), 652 calories per 24 hours. The basal metabolism of the wild rabbit is 6% higher than that of the domestic rabbit of the same average weight. At 16°C., the metabolism was 49% above the basal level.

## LITERATURE CITED

BENEDICT, F. G., AND J. M. PETŘÍK 1930 Metabolism studies on the wild rat. Am. J. Physiol., vol. 94, pp. 662–685.

LEE, R. C. 1939 a The rectal temperature of the normal rabbit. Am. J. Physiol., vol. 125, pp. 521–529.

———— 1939 b Basal metabolism of the adult rabbit and prerequisites for its measurement. J. Nutrition, vol. 18, pp. 473–488.

———— 1939 c Size and basal metabolism of the adult rabbit. J. Nutrition, vol. 18, pp. 489–500.

PURDY, W. J. 1925 Rabbits and experimental measles: blood-counts and rabbit temperatures. British J. Expt. Pathol., vol. 6, pp. 210–224.

# THE INFLUENCE OF VARIETY, SEASON AND GREEN MANURES UPON THE COMPOSITION OF WHEATS

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A wide variation has been found to occur in the ash, calcium, magnesium, potassium, iron, phosphorus and sulfur content of wheat collected from different parts of Utah (Greaves and Hirst, '29). This may be due in part to the variation in composition of the soil, yet the soils thus far examined contain large quantities of the essential mineral plant elements. Hence, other factors, such as, for example, the quantity of irrigation water applied during the growing season which has been shown to change materially the composition of wheat, oats, barley and corn (Greaves and Nelson, '25; Greaves and Carter, '23; Greaves and Hirst, '29), may play a predominating role. The present data are the results of a study of the influence of variety, season and manurial treatments upon the ash, calcium, magnesium, potassium, iron, sulfur and phosphorus content of wheats.

The wheats under consideration were grown on the Nephi dry-land substation located about 5 miles south of Nephi, Utah, on the north slope of the Levan Ridge. The soil is of the deep dry-farm type derived from the weathering of the adjacent mountain ranges and contains phosphates, potassium and large quantities of gypsum. The soil of the farm is a clay loam, and contains in the surface foot-section as an average 4100 pounds of nitrogen, 7000 pounds of phosphorus, 86,000 pounds of potassium, 1480 pounds of sulfur, 42,000 pounds of organic carbon, 69,000 pounds of magnesium carbonate, and 139,000 pounds of calcium carbonate. Consequently,

it is rich in these plant food elements. Water is the limiting factor of crop production, the average annual precipitation being 13 inches.

The results reported in this paper are from analyses of wheats grown on this soil. The samples were obtained as follows: Each yearly sample was composed of three replications of individual varieties. The samples were analyzed yearly for 8 years according to the methods of the Association of Official Agricultural Chemists ('24) for ash, calcium, magnesium, phosphorus and sulfur. The analyses for potassium cover a period of 3 years, and those of iron 5 years.

The results for ash, calcium, magnesium, iron and phosphorus for seven spring wheats are given in table 1. They are the averages of two determinations on each variety for each of 8 years. Variance was calculated by using the yearly percentages as replicates.

Although the differences between the varieties of wheat approach the point of significance, they cannot be judged significant on the basis of variation of the same wheat from year to year.

The results give a picture of the ash and ash constituents of these seven varieties of spring wheat when grown on a typical dry-farm soil.

The average composition of these spring dry-farm wheats is as follows: Ash, 1.58%; calcium, 0.071%; magnesium, 0.192%; potassium, 0.391%; iron 0.0058%; phosphorus, 0.298%; and sulfur, 0.213%.

The mineral composition of seventeen varieties of winter wheat, grown on the same soils and under similar conditions except that the seeding was done in the fall, is given in table 1.

A significant difference in these wheats was found in the calcium content, but the differences with respect to the other constituents are not significant, though they approach the 5% point. The average composition of these winter wheats follows: ash, 1.40%; calcium, 0.068%; magnesium, 0.157%;

potassium, 0.361%; iron 0.0064%; phosphorus, 0.276%; and sulfur, 0.188%.

The averages for the winter wheats are lower than those for the spring wheats. These results are of special importance in that we here have a measure of the comparison of spring and winter wheats. Approximately one-twentieth

TABLE 1

*Average percentages of ash, calcium, magnesium, potassium, iron, phosphorus and sulfur in varieties of wheats*

| | Ash | Ca | Mg | K | Fe[1] | P | S |
|---|---|---|---|---|---|---|---|
| | % | % | % | % | % | % | % |
| *Spring varieties* | | | | | | | |
| Regenerated defiance | 1.65 | 0.038 | 0.194 | 0.423 | 0.0056 | 0.296 | 0.207 |
| Kota | 1.60 | 0.081 | 0.205 | 0.364 | 0.0060 | 0.327 | 0.243 |
| Marquis | 1.60 | 0.076 | 0.177 | 0.387 | 0.0062 | 0.291 | 0.194 |
| Kubanka | 1.58 | 0.080 | 0.170 | 0.387 | 0.0058 | 0.315 | 0.204 |
| Early baart | 1.58 | 0.072 | 0.239 | 0.438 | 0.0054 | 0.287 | 0.211 |
| Chul | 1.54 | 0.072 | 0.163 | 0.368 | 0.0059 | 0.284 | 0.203 |
| Hard federation | 1.52 | 0.075 | 0.196 | 0.360 | 0.0060 | 0.288 | 0.231 |
| Average | 1.58 | 0.071 | 0.192 | 0.391 | 0.0058 | 0.298 | 0.213 |
| *Winter varieties* | | | | | | | |
| Utac | 1.71 | 0.085 | 0.178 | 0.408 | 0.0068 | 0.307 | 0.223 |
| Kofod | 1.53 | 0.060 | 0.169 | 0.421 | 0.0058 | 0.302 | 0.188 |
| Regal | 1.42 | 0.074 | 0.169 | 0.332 | 0.0070 | 0.289 | 0.170 |
| Tenmarq | 1.29 | 0.062 | 0.162 | 0.312 | 0.0055 | 0.284 | 0.177 |
| Montana no. 36 | 1.35 | 0.070 | 0.163 | 0.356 | 0.0058 | 0.282 | 0.179 |
| Sevier no. 34 | 1.46 | 0.068 | 0.158 | 0.406 | 0.0061 | 0.280 | 0.194 |
| Kanred | 1.45 | 0.068 | 0.178 | 0.361 | 0.0066 | 0.279 | 0.197 |
| Kharmont | 1.34 | 0.067 | 0.153 | 0.355 | 0.0068 | 0.277 | 0.198 |
| Kharkof | 1.36 | 0.065 | 0.169 | 0.347 | 0.0062 | 0.276 | 0.180 |
| Turkey and Kofod | 1.34 | 0.062 | 0.158 | 0.373 | 0.0060 | 0.273 | 0.197 |
| Sevier no. 59 | 1.43 | 0.066 | 0.133 | 0.366 | 0.0068 | 0.272 | 0.195 |
| Alton | 1.33 | 0.072 | 0.165 | 0.366 | 0.0056 | 0.271 | 0.170 |
| Black hull | 1.40 | 0.074 | 0.063 | 0.333 | 0.0062 | 0.268 | 0.188 |
| Kharkof Hayes no. 2 | 1.30 | 0.070 | 0.162 | 0.376 | 0.0064 | 0.268 | 0.174 |
| Turkey no. 926 | 1.32 | 0.059 | 0.160 | 0.362 | 0.0064 | 0.264 | 0.206 |
| Turkey | 1.36 | 0.072 | 0.170 | 0.353 | 0.0088 | 0.258 | 0.189 |
| Newturk | 1.42 | 0.068 | 0.153 | 0.304 | 0.0063 | 0.247 | 0.164 |
| Average | 1.40 | 0.068 | 0.157 | 0.361 | 0.0064 | 0.276 | 0.188 |

[1] All the samples of wheat were ground in an iron mill; consequently they would receive iron from this source. See E. B. Forbes, F. M. Bugle and J. E. Mensching ('13).

of the ash of these wheats is calcium and about one-ninth magnesium, or there is approximately twice as much magnesium as calcium.

These averages for ash, calcium and magnesium are slightly lower than those reported for wheats of the state of Utah (Greaves and Hirst, '29). This is due to the fact that the wheats here reported are all dry-farm wheats. The earlier data contained analyses of both dry-farm and irrigated wheats. Nevertheless the present results are higher than those given by Lawes and Gilbert (1884), Konig (1893) and Sherman ('37). They are higher in calcium and magnesium and lower in potassium, phosphorus and sulfur than Ohio wheats (Forbes, Bugle and Mensching, '13).

The calcium-phosphorus ratio of these wheats is approximately 1 to 4, thus indicating that a supplement high in calcium should be added when the wheats are fed. Alfalfa samples thus far analyzed show a calcium-phosphorus ratio of 6 to 1; hence it is highly probable that any legumes raised on highly calcareous soil make excellent supplements for these wheats.

The question naturally arises as to whether the differences which have been found in the mineral content of spring and winter wheats would manifest themselves if the wheats were fed to animals. This has been answered by Greaves and Greaves ('33) for Kota, a high calcium and phosphorus spring wheat, and Turkey, a low calcium and phosphorus winter wheat. These wheats were fed to young albino rats 22 to 25 days old for 3 weeks. The animals were kept in individual cages on a diet consisting of 90% wheat and 10% of the following mixture: 24 gm. casein, 32 gm. yeast, 12 gm. sodium chloride and 17 gm. cabbage. Fifteen replications were used in the case of the Kota and ten in the case of Turkey. The animals fed Kota made better gains and had better bones as shown by the higher percentages of ash, calcium and phosphorus. Moreover, the serum calcium and phosphorus in all animals fed Kota were higher than in those fed Turkey.

In order to learn if there is a relationship between the various ash constituents, correlations were made according to the formula given by Wallace and Snedecor ('31); they are presented in table 2.

The wheats were ground in an iron mill and only 1-year analyses were made of the iron. This may account for the absence of a positive correlation between iron and the ash and other ash constituents. However, there is a highly significant correlation between most of the other mineral constituents. Most of the sulfur (Greaves and Bracken, '37) and phosphorus of these wheats is in organic combinations; hence, the close relationship of these elements is shown in a

TABLE 2

*Correlations between the various constituents of twenty-four varieties of wheat. These figures are based upon the average quantities of material found in the wheat from 1923 to 1935*

| | Fe | P | Ca | K | S | Mg |
|---|---|---|---|---|---|---|
| P | −0.325[1] | | | | | |
| Ca | 0.007 | 0.551[1] | | | | |
| K | −0.233 | 0.479[1] | 0.281[2] | | | |
| S | −0.104 | 0.628[1] | 0.459[1] | 0.449[1] | | |
| Mg | −0.203 | 0.492[1] | 0.459[1] | 0.525[1] | 0.607[1] | |
| Ash | −0.218 | 0.718[1] | 0.721[1] | 0.626[1] | 0.651[1] | 0.602[1] |

[1] Highly significant.
[2] Significant.

highly significant correlation between the calcium of these wheats and the phosphorus, sulfur, magnesium and ash, but no significant correlation is observed between the calcium and potassium, and iron.

A significant correlation is indicated between the potassium of these wheats and the calcium, and a highly significant correlation between the potassium and the phosphorus, sulfur, magnesium and ash.

The sulfur content also varies directly with the quantity of the other constituents with the exception of iron. These wheats are especially high with respect to phosphorus and ash. The correlations in the cases of magnesium and ash and the

other constituents with the exception of iron are also highly significant. Hence it may be concluded that insofar as these dry-farm wheats are concerned, and tentatively insofar as the dry-farm wheats of the state are concerned, a high ash content indicates a high phosphorus, calcium, potassium, sulfur and magnesium content.

The twenty-six varieties were analyzed separately during each of 8 years, giving a good comparison for years. The average results obtained each year are given in table 3.

TABLE 3

*Average percentages of ash, calcium, magnesium, potassium, iron, phosphorus, and sulfur in twenty-four varieties of wheat during years 1923 and 1929–1935*

| CONSTITUENT | 1923 | 1929 | 1930 | 1931 | 1932 | 1933 | 1934 | 1935 |
|---|---|---|---|---|---|---|---|---|
| | % | % | % | % | % | % | % | % |
| Ash | 1.580 | 1.450 | 1.390 | 1.610 | 1.520 | 1.440 | 1.090 | 1.550 |
| Calcium | 0.083 | 0.069 | 0.075 | 0.066 | 0.068 | 0.065 | 0.070 | 0.069 |
| Magnesium | 0.199 | 0.173 | 0.181 | 0.171 | 0.202 | 0.169 | 0.142 | 0.150 |
| Phosphorus | 0.285 | 0.213 | 0.305 | 0.345 | 0.298 | 0.286 | 0.230 | 0.328 |
| Sulfur | 0.190 | 0.225 | 0.206 | 0.190 | 0.174 | 0.178 | 0.164 | 0.182 |
| Potassium | 0.389 | 0.359 | 0.385 | | | | | |
| Iron | 0.004 | 0.007 | 0.006 | 0.006 | 0.006 | | | |

A large variation is shown in the ash from year to year. In 1934 it was 1.09%, whereas in 1923 it was 1.58%, a difference of 45%. Calcium shows only slight variation, whereas magnesium varied 40%. The phosphorus and sulfur in these wheats both show a high yearly variation. The iron content shows very little, if any, variation. This may be due to the fact that the wheats were milled in an iron mill.

### *Influence of green manures*

During 8 years, Utah Kanred winter wheat was grown on soil which received treatments as indicated in table 4.

The data, averages of which are shown in table 4, were analyzed by variance and, as indicated, a highly significant difference was found for wheat yields as influenced by the various treatments. Similarly, a highly significant difference was found for percentage of calcium contained in the wheat.

For phosphorus the difference was only significant with odds of 20:1 that the variation was not due to chance alone. The percentages of ash, magnesium, and sulfur showed no definite trend.

It is interesting to note that the addition of green manures to these soils increased the phosphorus content of the wheat. This, in all probability, resulted from the fact that the soil

TABLE 4

*Average acre yields and average percentage composition with respect to ash, calcium, magnesium, phosphorus and sulfur of Utah Kanred wheat grown on soil receiving different manurial treatments*

| | AVERAGE YIELD PER ACRE | AVERAGE COMPOSITION | | | |
|---|---|---|---|---|---|
| | | Ash | Ca | Mg | P | S |
| | *bushels* | % | % | % | % | % |
| Fall plowed for fallow | 19.19 | 1.91 | 0.067 | 0.176 | 0.299 | 0.19 |
| Peas plowed when in pod | 16.52 | 1.71 | 0.068 | 0.180 | 0.302 | 0.18 |
| Peas plowed when in bloom | 15.35 | 1.62 | 0.070 | 0.175 | 0.313 | 0.18 |
| Peas plowed when 12 inches high | 16.19 | 1.55 | 0.072 | 0.184 | 0.317 | 0.19 |
| Peas plowed when 6 inches high | 17.92 | 1.60 | 0.068 | 0.188 | 0.291 | 0.19 |
| Wheat plowed when in milk | 12.23 | 1.70 | 0.071 | 0.193 | 0.314 | 0.16 |
| Wheat plowed when in bloom | 13.17 | 1.67 | 0.072 | 0.174 | 0.334 | 0.17 |
| Wheat plowed when 12 inches high | 14.90 | 1.59 | 0.069 | 0.182 | 0.331 | 0.17 |
| Wheat plowed when 6 inches high | 17.22 | 1.56 | 0.067 | 0.180 | 0.317 | 0.17 |
| Fall plowed for fallow | 18.45 | 1.52 | 0.063 | 0.179 | 0.287 | 0.19 |
| All straw plowed under | 18.92 | 1.53 | 0.062 | 0.171 | 0.299 | 0.18 |
| All straw burned | 19.65 | 1.58 | 0.065 | 0.191 | 0.278 | 0.18 |
| High headed straw plowed | 19.43 | 1.61 | 0.066 | 0.187 | 0.305 | 0.17 |
| High headed straw burned | 20.08 | 1.52 | 0.065 | 0.179 | 0.273 | 0.17 |
| Significant differences, Odds 20:1 | 1.85 | ... | 0.0089 | .... | 0.031 | ... |
| Highly significant difference, Odds 100:1 | 2.44 | ... | 0.0112 | .... | .... | ... |

carries an abundance of total phosphorus, but the quantity available during the growing season is limited by the weather, cultural treatment and the quantity of organic material decomposed. Inasmuch as the soil is low in organic matter the quantity decomposed each year is small, but when fresh organic material is added bacterial activity is increased with a resulting increase in the available phosphorus, which is reflected in the phosphorus content of the grain.

## CONCLUSIONS

Spring wheat grown on the Nephi substation dry farm carries greater percentages of ash, calcium, magnesium, potassium, iron, phosphorus and sulfur than does winter wheat grown on the same soil. A significant difference was found in the calcium content of different varieties of winter wheat grown on the same soil. A highly significant correlation was found to exist among most of the mineral constituents of wheat. When the wheats of different years were compared, a high variation was found to occur in the various mineral constituents from year to year. The addition of green manures to a typical dry farm soil caused a highly significant variation in the ash content and a significant difference with respect to calcium and phosphorus. The use of green manures materially increased the phosphorus content of wheat probably due to its effect in increasing bacterial activities which in turn increased the available plant food in the soil.

## LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1924 Official and tentative methods of analyses. Washington, D. C.

FORBES, E. B., F. M. BUGLE AND J. E. MENSCHING 1913 Mineral and organic analyses of foods. Ohio Agr. Expt. Sta. Bull. 255.

GREAVES, J. E., AND E. G. CARTER 1923 The influence of irrigation water on the composition of grains and the relationship to nutrition. J. Biol. Chem., vol. 58, pp. 531–541.

GREAVES, J. E., AND D. H. NELSON 1925 The influence of irrigation water and manure on the composition of the corn kernel. J. Agr. Research., vol. 31, pp. 103–189.

GREAVES, J. E., AND C. T. HIRST 1929 The mineral content of grains. Utah Exp. Sta. Bul. 210.

GREAVES, ETHELYN O., AND J. E. GREAVES 1933 Nutritive value of high and low calcium carrying wheats. J. Nutrition., vol. 6, pp. 113–125.

GREAVES, J. E., AND AARON F. BRACKEN 1937 The sulfur content of wheat. Cereal Chemistry, vol. 14, pp. 578–581.

KONIG, J. 1893 Der Meuchlichien Nabreengs und Genus-Smittel. Bd. II. Berlin.

LAWES, J. B., AND J. H. GILBERT 1884 On the composition of the ash of wheat grain and wheat straw grown at Rothamsted in different seasons and by different manures. J. Chem. Soc., vol. 45, p. 305.

SHERMAN, H. C. 1937 Chemistry of food and nutrition. 5th ed., rev. Macmillan Co., New York.

WALLACE, H. A., AND GEORGE W. SNEDECOR 1931 Correlation and machine calculations. Collegiate Press, Ames, Iowa.

# IRON METABOLISM

## 1. THE ROLE OF CALCIUM IN IRON ASSIMILATION [1]

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A conviction, apparently widely shared by those engaged in nutritional research, attributes to calcium the faculty of increasing iron absorption from the intestinal tract or in otherwise aiding in its economy. It is based, as nearly as the author can determine, on the initial work of von Wendt ('05) and that of Sherman ('07). The study to be reported here yielded contrary findings.

The observation that an anemia developed in a high-tumor strain of mice fed a diet consisting wholly of a commercial dog biscuit led to this work. An analysis of this diet revealed the presence of an unusually high percentage of calcium, amounting to more than 3½%. Since this product constituted the sole article of diet for mice in these laboratories over a period of years, the question arose as to what, if any, the relationship of the calcium to the observed anemia might be, and whether or not this relationship contributed to a larger syndrome which terminated in the production of new growths.

That an iron-calcium antagonism was involved in the production of the anemia in our mice was further suggested by certain results of Rose and Vahlteich ('32). They found that when adequate and similar amounts of iron and copper in the form of whole wheat grain or as the ash of the same supplemented with milk were fed to anemic rats, lower hemoglobin

[1] Portions of these data were presented before the American Chemical Society, Cleveland, Ohio, September, 1934, and before the American Institute of Nutrition, Detroit, Michigan, April, 1935.

readings were obtained when the iron and copper were administered in the form of the ash than in the form of the whole grain. No explanation was offered for their observations. It occurred to us that these observed differences might be explained on the basis of the greater amount of milk consumed and the correspondingly greater calcium intake in the case of the animals receiving their iron and copper as part of the ash of the whole wheat.

Both von Wendt ('05) and Sherman ('07) determined iron retention in human adult subjects by means of balance experiments. Their experimental periods were a matter of days. Because of the shortcomings inherent in an experimental procedure of this nature, we decided to use rats and to determine the total iron retention as revealed by chemical analysis of the entire animal after a period of 5 to 6 weeks of controlled feeding. A simple basal diet was used and the only variable was the addition of various supplements of calcium salts.

## EXPERIMENTAL PART

Basal diet 1 had the following percentage composition: ground yellow corn (ball-milled) 42.5; wheat flour [2] 42.5; commercial casein [3] 9; dried yeast [4] 3; cod liver oil 2; sodium chloride, C.P., 1. Ferric citrate U.S.P.[5] was added to make the iron content equal to 50 mg. per kilo. Rations were weighed out daily and were equalized, insuring an average 10 gm. daily intake for the duration of the feeding period. This diet provided a daily intake of 0.5 mg. of iron and 0.02 mg. of copper per animal.

In an attempt to determine the effect of calcium supplementation on a whole wheat ration, the following diet was used: ground whole wheat (ball-milled) 90%; commercial casein 5; dried yeast 2; cod liver oil 2; and sodium chloride, C.P., 1%. The calcium supplements were introduced as sub-

[2] Patent flour (Pillsbury XXXX).
[3] Crude casein from the Casein Mfg. Co. of America, Bainbridge, N. Y.
[4] Powdered yeast foam tablets from the Northwestern Yeast Co., Chicago, Ill.
[5] U. S. P. VIII-Scales (Merck) ball-milled.

stitutes for equivalent amounts of wheat. A 10 gm. daily intake of the diet furnished 0.5 mg. of iron and 0.07 mg. of copper.

The rats were of the Wisconsin and the large Yale albino strains. At 20 to 22 days they were placed on a dried whole milk [6] diet. When their hemoglobin values had reached a level of 4 gm. or less per 100 cc. of blood, which required from 2 to 3 weeks, representative animals were sacrificed for anemic controls and the remaining rats were distributed equally for the feeding of the basal and experimental diets.

All animals were subjected to exsanguination before their tissues were separated and prepared for analysis. This was effected by perfusion with Locke's solution under ether anaesthesia, using a technic which was adapted from the methods of Whipple ('26) and Bethke, Steenbock and Nelson ('23). The blood and perfusate were collected on specially prepared sponges of hospital gauze which had been prepared by extraction with dilute HCl and subsequent washing with distilled water. Analyses showed either that they were free of iron or that only negligible amounts were present. The degree of success attained in our exsanguination procedure was determined by expressing the total blood iron values in terms of grams of hemoglobin, and with the aid of blood volume data from Donaldson's monograph ('15) stating the hemoglobin in terms of grams per 100 cc. of blood. These values were in turn compared with those for hemoglobin determined by the Newcomer method, using a standardized Bausch and Lomb filter, the blood for this purpose having been obtained from the tail immediately before the sacrifice of the animal. Using such a criterion as a means of comparison, it appears that we were able to remove 90% and upward of all blood. Essential to a good perfusion are light anaesthesia, continued respiratory movements, and the introduction of from 25 to 40 cc. of saline solution, the latter depending upon the size of the animal. The significance of the removal of the blood from the solid tissues is evident when it is recalled that

[6] Klim.

blood constitutes only 6 to 8% of the weight of the animal, yet it contains from 55 to 65% of the total body iron. Following exsanguination, the liver and the spleen were removed, sliced, washed in physiological saline, weighed, and dried on watch glasses in an electric oven at 100°C. and then stored in glass containers until analyzed. The intestinal tract was removed, freed of all residues, washed, and added to the carcass, which was then dried in a manner similar to the liver and the spleen. The sponges were placed on watch glasses and dried over radiators with the necessary precautions.

Iron determinations were made according to a modified Elvehjem procedure ('30) for substances high in phosphorus. All ignitions were effected in quartz evaporating dishes by means of Meeker burners or in an electric muffle furnace. Prolonged heating on a boiling water bath and on electric hot plates according to Eggleton and Eggleton ('29) without the addition of alkali effected hydrolysis of pyrophosphates. Color fading was not experienced under our operating procedures and quantitative recovery of added iron was the rule.

## RESULTS

The results of our initial experiment involving the addition of 1% of $CaCO_3$ to the basal diet are given in table 1. The carcass iron includes that of all organs except those specifically mentioned in the table.

An analysis of the results reveals that the addition of 1% of $CaCO_3$ has reduced the iron content of all tissues except the spleen, the average liver, blood, and carcass values being 57, 86 and 90% of the respective control values. The livers of the calcium-supplemented animals average less than the livers of the anemic controls sacrificed at the beginning of the experimental feeding.

In table 1 are also recorded the results of feeding 3% of $CaCO_3$. The accentuated effect of increased calcium supplementation is evidenced by the fact that the average liver, blood, and carcass values are only 28, 72 and 61% of the respective control values.

TABLE 1

*The effect on total iron retention by rats of the addition of calcium carbonate to the basal diet over a period of 5 weeks*

| DIET | ANIMAL | | IRON CONTENT | | | |
|---|---|---|---|---|---|---|
| | No. | Body weight | Liver | Spleen | Blood | Carcass |
| | | gm. | mg. | mg. | mg. | mg. |
| Basal only | 141 | 150 | 0.640 | 0.110 | 3.658 | 1.870 |
| | 143 | 168 | 1.010 | 0.138 | 3.740 | 2.620 |
| | 145 | 175 | 0.624 | 0.093 | 4.064 | 2.245 |
| | 147 | 226 | 0.535 | 0.117 | 4.675 | 2.730 |
| | 149 | 196 | 0.762 | 0.107 | 4.275 | 2.300 |
| Average | | 183 | 0.714 | 0.107 | 4.168 | 2.286 |
| Basal plus 1% of $CaCO_3$ | 142 | 155 | 0.356 | 0.096 | 3.280 | 1.950 |
| | 144 | 170 | 0.374 | 0.094 | 3.517 | 1.845 |
| | 146 | 205 | 0.444 | 0.111 | 3.794 | 2.245 |
| | 148 | 203 | 0.454 | 0.119 | 3.781 | 2.175 |
| Average | | 183 | 0.407 | 0.105 | 3.593 | 2.053 |
| Anemic controls (4) | | 104 | 0.459 | 0.083 | 0.862 | 0.785 |
| Basal only | 441 | 163 | 1.130 | | 3.662 | 2.595 |
| | 443 | 155 | 0.412 | Included | 3.594 | 2.550 |
| | 445 | 200 | 0.472 | with | 4.347 | 3.310 |
| | 447 | 185 | 0.560 | carcass | 3.328 | 2.570 |
| | 449 | 165 | 0.485 | | 3.507 | 2.355 |
| Average | | 173 | 0.611 | | 3.687 | 2.676 |
| Basal plus 3% of $CaCO_3$ | 442 | 173 | 0.218 | | 2.968 | 1.865 |
| | 444 | 178 | 0.153 | | 2.798 | 1.820 |
| | 446 | 156 | 0.194 | | 2.085 | 1.720 |
| | 448 | 175 | 0.151 | | 3.052 | 1.645 |
| | 450 | 146 | 0.171 | | 2.654 | 1.405 |
| | 451 | 160 | 0.166 | | 2.315 | 1.345 |
| Average | | 164 | 0.175 | | 2.645 | 1.633 |
| Anemic controls (4) | | 104 | 0.134 | | 0.533 | 0.438 |
| Basal only | 4413 | 200 | 0.310 | Included with carcass | 4.241 | 2.730 |
| | 4415 | 201 | 0.362 | | 4.108 | 2.525 |
| | 4417 | 161 | 0.546 | | 3.231 | 2.100 |
| | 4419 | 177 | 0.770 | | 3.837 | 2.210 |
| Average | | 186 | 0.497 | | 3.604 | 2.391 |
| Basal plus calcium lactate equivalent to $2\frac{1}{2}$% of $CaCO_3$ | 4414 | 141 | 0.139 | | 1.523 | 1.266 |
| | 4416 | 148 | 0.123 | | 1.966 | 1.190 |
| | 4418 | 108 | 0.115 | | 1.090 | 0.896 |
| | 4420 | 145 | 0.116 | | 1.454 | 0.976 |
| Average | | 135 | 0.123 | | 1.508 | 1.082 |
| Anemic controls (5) | | 76 | 0.166 | | 0.774 | 0.607 |

The results of feeding calcium lactate in amounts equivalent to 2½% of $CaCO_3$ are given in table 1. These indicate that the depressive effect of calcium is increased when calcium is supplied in this form. In this case the average liver, blood and carcass values are only 25, 42 and 45% of the respective control values.

The results of feeding $CaCl_2$, $Ca_3(PO_4)_2$, and $CaSO_4$ in amounts equivalent in calcium content to 3% of $CaCO_3$

TABLE 2

*The effect on total iron retention by rats of the addition of calcium chloride, tri-calcium phosphate, and calcium sulphate equivalent in calcium content to 3% of calcium carbonate over a period of 5 weeks*

| DIET | ANIMAL | | IRON CONTENT | | | |
|---|---|---|---|---|---|---|
| | Litter no. | Body weight | Liver | Spleen | Blood | Carcass |
| | | gm. | mg. | mg. | mg. | mg. |
| Basal | 1 | 163 | 1.000 | 0.082 | 3.774 | 2.064 |
| only | 2 | 178 | 1.227 | 0.120 | 3.424 | 2.056 |
| | 3 | 165 | 0.578 | 0.112 | 3.390 | 1.992 |
| | 4 | 187 | 1.110 | 0.072 | 3.520 | 2.880 |
| Average | | 173 | 0.979 | 0.096 | 3.527 | 2.243 |
| Basal | 1 | 82 | 0.206 | 0.089 | 1.892 | 1.104 |
| plus | 2 | 57 | 0.127 | 0.093 | 1.318 | 1.010 |
| $CaCl_2$ | 3 | 57 | 0.093 | 0.101 | 1.285 | 0.796 |
| | 4 | 135 | 0.110 | 0.103 | 2.041 | 1.220 |
| Average | | 83 | 0.134 | 0.096 | 1.659 | 1.032 |
| Basal | 1 | 126 | 0.163 | 0.095 | 2.221 | 1.292 |
| plus | 2 | 155 | 0.220 | 0.122 | 3.200 | 1.824 |
| 3% of | 3 | 159 | 0.222 | 0.148 | 3.365 | 1.724 |
| $CaCO_3$ | 4 | 165 | 0.288 | 0.077 | 3.422 | 2.236 |
| Average | | 151 | 0.223 | 0.110 | 3.052 | 1.769 |
| Basal | 1 | 141 | 0.148 | 0.092 | 2.755 | 1.580 |
| plus | 2 | 184 | 0.396 | 0.214 | 4.117 | 1.940 |
| $Ca_3(PO_4)_2$ | 3 | 148 | 0.479 | 0.145 | 3.372 | 1.600 |
| | 4 | 170 | 0.199 | 0.070 | 3.077 | 2.200 |
| Average | | 161 | 0.305 | 0.130 | 3.330 | 1.830 |
| Basal | 1 | 130 | 0.769 | 0.071 | 4.562 | 1.856 |
| plus | 2 | 162 | 0.610 | 0.120 | 3.397 | 2.000 |
| $CaSO_4$ | 3 | 146 | 0.962 | 0.301 | 3.471 | 2.012 |
| | 4 | 148 | 0.806 | 0.082 | 3.565 | 2.176 |
| Average | | 147 | 0.787 | 0.143 | 3.749 | 2.011 |
| Anemic controls (7) | | 66 | 0.360 | 0.081 | 1.262 | 0.706 |

(table 2) show that these other calcium salts with the exception of $CaSO_4$ have a similar adverse effect on iron retention. The unpalatability of the $CaCl_2$ diet reduced consumption by 25%. The remaining animals were maintained on a 10 gm. daily intake.

TABLE 3

*The effect on total iron retention by rats of the addition of calcium carbonate to a whole wheat diet over a period of 60 days*

| DIET | HEMOGLOBIN PER 100 CC. | | ANIMAL | | IRON CONTENT | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Body weight | Litter no. | Liver | Spleen | Blood | Carcass |
| | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* | *mg.* |
| Wheat | 2.03 | 12.51 | 165 | 1 | 0.707 | 0.200 | 4.528 | 3.795 |
| diet | 2.65 | 12.05 | 165 | 2 | 1.117 | 0.290 | 4.315 | 2.890 |
| only | 3.05 | 14.20 | 145 | 3 | 0.884 | 0.159 | 4.174 | 2.275 |
| | 3.05 | 14.90 | 185 | 4 | 0.612 | 0.159 | 4.520 | 3.125 |
| | 2.72 | 12.77 | 164 | 5 | 0.946 | 0.196 | 4.434 | 2.680 |
| Average | 2.70 | 13.28 | 165 | | 0.853 | 0.201 | 4.394 | 2.953 |
| Wheat | 2.29 | 12.77 | 161 | 1 | 0.738 | 0.257 | 4.525 | 1.845 |
| diet | 2.71 | 10.71 | 165 | 2 | 0.724 | 0.266 | 3.715 | 2.295 |
| plus | 3.47 | 11.29 | 158 | 3 | 0.589 | 0.140 | 4.207 | 2.330 |
| 1% of | 3.59 | 12.05 | 175 | 4 | 0.887 | 0.284 | 4.515 | 2.325 |
| $CaCO_3$ | 3.06 | 13.47 | 159 | 5 | 0.634 | 0.186 | 4.252 | 2.184 |
| Average | 3.02 | 12.06 | 164 | | 0.714 | 0.227 | 4.241 | 2.196 |
| Wheat | 2.29 | 13.47 | 155 | 1 | 0.692 | 0.135 | 4.431 | 2.845 |
| diet | 2.89 | 11.59 | 143 | 2 | 0.521 | 0.094 | 3.692 | 1.885 |
| plus | 3.87 | 12.05 | 165 | 3 | 0.579 | 0.107 | 4.305 | 2.295 |
| 3% of | 4.89 | 13.19 | 171 | 4 | 0.798 | 0.156 | 4.611 | 2.655 |
| $CaCO_3$ | 3.85 | 12.77 | 180 | 5 | 0.380 | 0.148 | 4.564 | 2.080 |
| Average | 3.56 | 12.61 | 163 | | 0.594 | 0.128 | 4.321 | 2.352 |
| Anemic controls (5) | | 4.17 | 97 | | 0.586 | 0.059 | 0.883 | 0.960 |

In table 3 are recorded the results of calcium supplementation of a modified whole wheat diet over a period of 60 days. Starting with the lowest initial hemoglobin readings, the unsupplemented-wheat animals show the highest average hemoglobin regeneration and the highest average iron values for carcass, blood and liver. The calcium-supplemented animals retained only 82.5% of the iron of the unsupplemented-wheat animals. The differences between the 1 and 3% $CaCO_3$ groups are less obvious; blood regeneration was equally good;

while the iron values for liver and spleen of the 1% $CaCO_3$ group were superior to those of the 3% $CaCO_3$ group, the reverse was true for the blood and carcass values.

The data as analyzed thus far, with the exception of $CaSO_4$, point to the conclusion that under the conditions which have been described, calcium exerts an adverse effect on iron assimilation. Klètzien ('35) previously had held that $CaSO_4$, too, acts to diminish iron assimilation; later and more extensive observations do not substantiate this. The depressive effect is likewise evident in the case of the citrate, acetate, tartrate, and malate of calcium, the data of which will be incorporated in a paper bearing on the effect of anions on the iron assimilatory process. Evidence has also been accumulated indicating that calcium oxalate acts like the sulphate. In these experiments, with the exceptions noted, the initial milk-induced anemia was not corrected, or only partially so, by diets supplemented with calcium, in spite of an adequate iron and copper intake. It seems reasonable to regard the anemia in our mice as due to the high calcium content of the commercial dog biscuit, which constituted their sole dietary. The results of Rose and Vahlteich ('32) could be explained on a similar basis.

## DISCUSSION

An analysis of our tissue iron values seems to indicate that in the animal economy, the synthesis of hemoglobin predominates over that of other iron-containing respiratory pigments, suggesting the possibility of a deficiency of the latter, together with disturbed oxygen relationships, even though the blood picture is not obviously that of anemia.

From the comparative studies of Macallum ('26) on the mineral constituents of tissue fluids and sea water, and the studies of Johnston and Ball ('30), Ravdin et al. ('32), and de Beer et al. ('35) on the mineral composition of the secretions of the alimentary tract, it is suggested that in the present investigation we are dealing with a disturbance in the normal concentration of the common inorganic ions in the principal

absorptive portions of the digestive tract. That the introduction of readily ionizable or metabolizable salts of calcium within the intestinal tract may provoke an ionic imbalance with concomitant disturbances within or at the protoplasmic boundaries does not seem to be mere idle speculation. This may serve to explain the results in table 2 where the use of $CaCl_2$ gave effects different from those yielded by $CaSO_4$. The corrosive effects of $CaCl_2$ are probably not due to its composition as such, but are related rather to its ready solubility and high degree of ionization, overcoming thereby the ionic antagonism of the Na and K and interfering with the normal reactions involving chlorides, carbonates, and phosphates. That adsorption is a factor in making iron unavailable is not disproved; neither are changes in intestinal pH or the removal of iron by precipitation.

Gardner and Burget ('38) observed that KCl added to glucose solutions increased the absorption of glucose from chronic closed intestinal loops in dogs; the contrary was observed when $CaCl_2$ was introduced in similar concentrations. These results confirmed in certain respects earlier work of Magee and Sen ('32) and Gellhorn and Skupa ('33). Robscheit- Robbins and her co-workers ('28) pointed to "salts as perhaps responsible" for the marked superiority of apricots for hemoglobin regeneration. Insofar as the iron and copper required for hemoglobin regeneration are concerned, this explanation is very plausible, in view of the marked preponderance of K and Na in apricots in contradistinction to their content of Ca and Mg.

That the retention of copper is affected by calcium supplementation in a manner similar to that of iron is suggested by other unpublished data of ours. The association of deficiencies of other minor inorganic elements such as iodine, manganese, cobalt, zinc and boron with excessive calcium supplementation is suggested. Reference to iodine in this connection was made by Kletzien ('34) in an explanation of results of Levine, Remington and v. Kolnitz ('33). The identification of perosis (slip tendon disease of fowl) with

manganese deficiency by Wilgus et al. ('36) and the known high incidence of this disease with use of diets heavily supplemented with calcium may also be cited as evidence for such an association. The occurrence of certain "salt lick" diseases of animals subsisting on vegetation grown on soils of a calcareous origin and their correction by fertilizing with traces of these minor elements offers additional corroborative evidence. Similarly, the identification of so-called "goitre belts" with regions having a history of limestone origin may be cited.

## SUMMARY

1. Young rats were fed a milk diet productive of a severe anemia, following which the effect of variation in the supply of calcium on the amount of iron in the various organs and the entire body was studied.

2. The addition of 1 and 3% of calcium carbonate to the basal diet resulted in lower tissue iron values. This was also true when calcium in the form of the lactate equivalent to 2½% and the chloride and tri-phosphate equivalent to 3% of calcium carbonate were fed. This was not true, however, in the case of calcium sulphate.

3. The addition of 1 and 3% of calcium carbonate to a basal diet containing 90% of ground whole wheat resulted in lower tissue iron values.

4. The possible bearing of these findings on the metabolism of other minor elements is discussed.

## LITERATURE CITED

Bethke, R. M., H. Steenbock and Mariana T. Nelson 1923 Calcium and phosphorus relations to growth and composition of blood and bone with varying vitamin intake. J. Biol. Chem., vol. 58, p. 71.

de Beer, E. J., C. G. Johnston and D. W. Wilson 1935 The composition of intestinal secretions. J. Biol. Chem., vol. 108, p. 113.

Donaldson, H. H. 1915 The Rat, 1st ed. Memoirs of the Wistar Institute of Anatomy and Biology. No. 6.

Eggleton G. P., and P. Eggleton 1929 A method of estimating phosphagen and some other phosphorus compounds in muscle tissue. J. Physiol., vol. 68, p. 193.

ELVEHJEM, C. A. 1930 A note on the determination of iron in milk and other biological materials. J. Biol. Chem., vol. 86, p. 463.

GARDNER, J. W., AND G. E. BURGET 1938 The influence of calcium and potassium upon intestinal absorption. Am. J. Physiol., vol. 121, p. 475.

GELLHORN, E., AND A. SKUPA 1933 The K–Ca antagonism in regard to absorption from the intestine. Am. J. Physiol., vol. 106, p. 318.

JOHNSTON, C. G., AND E. G. BALL 1930 Variations in inorganic constituents of pancreatic juice during constant drainage of the pancreatic ducts. J. Biol. Chem., vol. 86, p. 643.

KLETZIEN, S. W. 1934 Calcium—its role in iron metabolism. Abstr. Div. of Biol. Chem. A. C. S. Meetings, Cleveland, Ohio.

———— 1935 The influence on iron assimilation of some of the elements in groups 1 and 2 of the periodic system. J. Nutition, vol. 9, supp. 9.

LEVINE, H., R. E. REMINGTON AND H. VON KOLNITZ 1933 A dietary technique for the study of goitre in the rat. J. Nutrition, vol. 6, p. 325.

MACALLUM, A. B. 1926 The paleochemistry of the body fluids and tissues. Physiol. Reviews, vol. 6, p. 316.

MAGEE, H. E., AND K. C. SEN 1932 The influence of electrolytes on the function of the intestinal mucosa. J. Physiol., vol. 75, p. 433.

RAVDIN, I. S., C. G. JOHNSTON, C. RIEGEL AND S. L. WRIGHT, JR. 1932 The anion-cation content of hepatic and gall-bladder bile. Am. J. Physiol., vol. 100, p. 317.

ROBSCHEIT-ROBBINS, F. S., C. A. ELDEN, W. M. SPERRY AND G. W. WHIPPLE 1928 Potent influence of inorganic ash of apricots, liver, kidney, and pineapple. J. Biol. Chem., vol. 79, p. 563.

ROSE, M. S., AND E. MCC. VAHLTEICH 1932 Factors in food influencing hemoglobin regeneration. 1. Whole wheat flour, white flour, prepared bran, and oatmeal. J. Biol. Chem., vol. 96, p. 593.

SHERMAN, H. C. 1907 Iron in food and its functions in nutrition. Office of Expt. Stations, U. S. D. A. Bull. no. 185.

VON WENDT, G. 1905 Untersuchungen über den Eiweiss- und Salz-Stoffwechsel beim Menschen. Skand. Arch. Physiol., vol. 17, p. 211.

WILGUS, H. S., JR., L. C. NORRIS AND G. F. HEUSER 1936 The role of certain inorganic elements in the cause and prevention of perosis. Science, N. S., vol. 84, p. 252.

WHIPPLE, G. H. 1926 The hemoglobin of striated muscle. I. Variations due to age and exercise. Am. J. Physiol., vol. 76, p. 693.

# THE PROTEIN CONTENT OF THE ORGANS AND TISSUES AT DIFFERENT LEVELS OF PROTEIN CONSUMPTION [1]

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TWO FIGURES

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The effect of variation in protein intake on the total quantity of protein in the body has been estimated from the nitrogen content of the body by Forbes, Voris, Bratzler and Wainio ('38) and by Hamilton ('39). However, the observations of Addis, Poo and Lew ('36) as to the individual manner in which different organs respond to fasting and protein refeeding make it quite likely that these total changes are only the sum of a series of quite dissimilar reactions. If that be the case, it becomes a matter of some practical importance as well as of general interest to determine whether different levels of protein intake are associated with differences in the distribution of total protein within the body (organ protein per 100 gm. total protein).

From a colony that had been reared on a diet containing 18% of protein, 120 rats about 90 days of age were selected. They were divided into six groups of twenty rats each so that the average body weight of each group was 150 gm. One of these groups was immediately sacrificed and the protein content of the organs and tissues determined, using the methods described by Addis, Poo, Lew and Yuen ('36). The other five groups were given diets that contained 6, 11, 16, 27 and 43%

[1] This work was aided by a grant from the Rockefeller Foundation.

of protein, respectively. These diets were almost exactly isocaloric since the protein variation was obtained by substituting casein for cornstarch. An air-dry commercial brand of casein was used; it contained 79.2% of protein. Each diet consisted of 60% by weight of a casein cornstarch mixture with the concentration of casein ranging from 4% at the lowest level through 10, 16 and 30 to 50% for the diet richest in protein. In addition each diet contained in per cent lard 15, sardine oil 10, dry yeast 9, air-dry ground alfalfa 2, and Osborne and Mendel's salt mixture 4%. After 18 days on these diets the protein content of the organs and tissues of each of the five groups was determined and compared with the amounts found in the group in which the measurements were made at 0 days. The results are given in table 1.

Since, at the start, each of the six groups had the same average body weight and represented samples chosen at random from the same population, we can obtain the quantities of protein newly formed in growth over a period of 18 days on these five levels of protein intake by subtracting the amounts found at 0 days from those found after 18 days. In order to facilitate relative comparison, the changes in total protein and in the protein content of the kidney, liver and blood serum have been expressed as percentages of the original quantities found at the start and plotted in figure 1 against the quantities of food protein in the 18-day period.

The examples given in figure 1 are sufficient to show that when protein consumption rises from amounts not even sufficient for maintenance through quantities adequate for growth to luxus levels there is a diversity in the effect produced on the various organs of the body. Thus, the kidney protein continues to rise with every increase in protein consumption but the maximum addition of protein to the liver is attained on the 27% protein diet, while with serum protein the greatest content is reached on 16% protein ration. A consideration of the results given in table 1 will show that this is also true for the other organs and tissues, so that the effect on the total body protein, which reaches its maximum at the mid-point in

TABLE 1

*Fresh weight and protein content per rat of organs and tissues before and after 18 days of increasing protein consumption*

| DIET | | | ORGAN[1] AND TISSUE EXAMINATION MADE | ORGANS AND TISSUES EXAMINED | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Protein content | Protein eaten in 18 days | | Kidney | Heart | Liver | Alimentary tract, etc. | Clot | Serum | Uterus | Carcass | Total |
| | % | gm. | | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| 8[2] | 18 | ... | Weight | 0.995 | 0.519 | 6.567 | 25.701 | 2.452 | 2.876 | 0.290 | 110.6 | 150.0 |
| | | | Protein | 0.161 | 0.083 | 1.256 | 1.631 | 0.712 | 0.166 | 0.040 | 19.497 | 23.546 |
| 1 | 6 | 9.5 | Weight | 0.853 | 0.545 | 5.435 | 22.750 | 2.150 | 2.341 | 0.223 | 108.6 | 142.9 |
| | | | Protein | 0.142 | 0.086 | 0.959 | 1.510 | 0.610 | 0.139 | 0.032 | 20.397 | 23.874 |
| 2 | 11 | 17.8 | Weight | 0.950 | 0.585 | 6.656 | 24.147 | 2.454 | 2.850 | 0.358 | 122.7 | 160.7 |
| | | | Protein | 0.158 | 0.097 | 1.229 | 1.658 | 0.692 | 0.186 | 0.049 | 22.619 | 26.687 |
| 3 | 16 | 27.1 | Weight | 1.092 | 0.622 | 7.398 | 28.083 | 2.884 | 3.215 | 0.358 | 133.3 | 177.0 |
| | | | Protein | 0.173 | 0.105 | 1.406 | 1.705 | 0.838 | 0.215 | 0.049 | 22.963 | 27.455 |
| 4 | 27 | 43.8 | Weight | 1.165 | 0.619 | 7.367 | 27.200 | 2.841 | 3.003 | 0.389 | 135.4 | 178.0 |
| | | | Protein | 0.189 | 0.100 | 1.454 | 1.746 | 0.819 | 0.199 | 0.055 | 23.603 | 28.166 |
| 5 | 43 | 80.0 | Weight | 1.191 | 0.534 | 6.595 | 26.400 | 2.571 | 2.661 | 0.355 | 125.1 | 165.4 |
| | | | Protein | 0.195 | 0.091 | 1.353 | 1.762 | 0.781 | 0.180 | 0.051 | 22.770 | 27.184 |

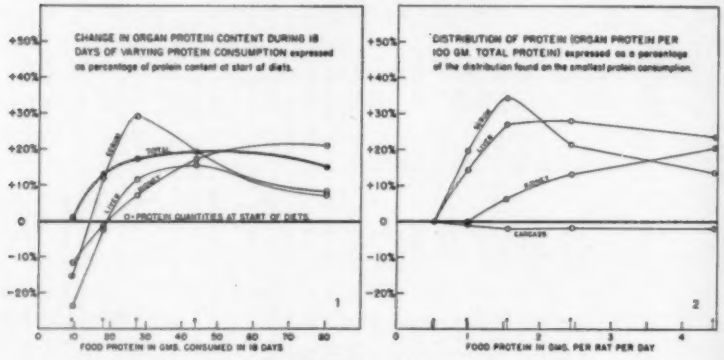
[1] Definitions of these organs and tissues as used in this study are as follows: 'Total Protein' includes all the protein of the rat, even that of the hair, but not the protein of the food within the alimentary tract. 'Kidney' is both kidneys stripped of their capsules, split open with a razor blade and blotted on filter paper for removal of blood and urine. 'Heart' is the organ removed in a uniform manner by section of the large vessels close to the muscle, and after removal of blood from the interior of the auricles and ventricles. 'Liver' is the organ after blotting on filter paper to remove surface blood. 'Alimentary Tract, etc.' includes the gastrointestinal canal from the diaphragm to close to the anus, the pancreas, spleen, adrenals, ovaries, mesentery, and bladder, and includes all the fat of the abdominal and pelvic cavities. After boiling in 0.5 molar sodium acetate solution at pH 5 the gastrointestinal tract is opened with scissors and the contents removed by washing under running water. 'Clot' and 'Serum' were separated by centrifuging at high speed for 30 minutes all the blood that could be obtained after cutting the abdominal aorta of the anesthetized rat while the heart was still beating. In a healthy rat about 75% of the total circulating blood volume is thus obtained. 'Uterus' includes the uterine horns stripped of fat and cut at the entrance into the vagina. Fluid contents are removed before weighing. 'Carcass' is the whole rat after exsanguination and removal of the heart and all abdominal and pelvic organs. This remainder consists predominantly of the muscle, skin and skeleton although the protein of the lungs, nervous system and peripheral vascular system is also included. 'Total Weight' is the live body weight and includes the contents of the gastrointestinal tract. 'Alimentary Tract etc. Weight' includes the weight of the gastrointestinal contents.

The protein quantities in the group killed before the diets were started were published in a previous paper (Poo, Lew and Addis, '39). It should be noted that in the fresh weights for this group given in that paper there is an error due to transposing part of the carcass weight to the weight of the alimentary tract, etc.

[2] Stock diet on which the animals subsisted before being placed on experiment.

the range of protein consumption, is only the sum of a series of quite individual organ and tissue reactions.

The concentration of protein in the organs and tissues (protein per 100 gm., fresh weight) can be obtained from the protein quantities and organ weights given in table 1. These concentrations vary in magnitude from 6.1% for the alimentary tract, etc. (where the protein was diluted by the abdominal pelvic fat), to 30.8% for blood clot. In the carcass and alimentary tract, etc., the protein concentrations seem to vary inversely with the amount of visible fat, for they are lowest on the 16% protein diet, where fat deposition is at a maximum,


CHANGE IN ORGAN PROTEIN CONTENT DURING 18 DAYS OF VARYING PROTEIN CONSUMPTION expressed as percentage of protein content at start of diets.
+50%
+40%
+30%
+20%
+10%
0
-10%
-20%
SERUM
TOTAL
LIVER
KIDNEY
0-PROTEIN QUANTITIES AT START OF DIETS
1
0 10 20 30 40 50 60 70 80
FOOD PROTEIN IN GMS. CONSUMED IN 18 DAYS
DISTRIBUTION OF PROTEIN (ORGAN PROTEIN PER 100 GM. TOTAL PROTEIN) expressed as a percentage of the distribution found on the smallest protein consumption.
SERUM
LIVER
KIDNEY
CARCASS
2
0 1 2 3 4
FOOD PROTEIN IN GMS. PER RAT PER DAY

Figures 1 and 2

and rise progressively in the leaner groups that were given either too little or too much protein.

In the serum and blood clot, and in the heart, kidney and uterus, there is no regular change in concentration with change in protein consumption. On the other hand, in the liver, where glycogen as well as fat and water is an appreciable part of the total weight, the protein concentrations change with each increment of food protein, increasing from 17.7% on the 6% ration to 20.5% on the 43% protein diet.

The distribution of organ protein (organ protein per 100 gm. total protein) is a measure that is independent of varia-

tion in the fat and water content or in the total body weight of the groups we are comparing. Under the conditions we have chosen, we pass from a supply of food protein so restricted that protein catabolism exceeds anabolism, through two gradations of adequate protein consumption to an amount so excessive that the rate of anabolism again becomes diminished. Under these diverse circumstances, each organ and tissue is in competition with all the others for its share of the total available protein, and alterations in the proportion that each in the end obtains, may be taken as an index to its relative functional importance in the total economy, if it is accepted that the protein content of an organ is the best available measure of its total operative metabolic machinery. These distributions can be deduced from the protein quantities given in table 1. The relative change in the proportions of the total protein allocated to various organs and tissues is shown in figure 2, where the distribution found on the lowest protein diet is taken as 100% and the distributions found at successively higher levels of protein consumption are expressed as a percentage of these quantities.

Figure 2 shows that as the protein intake increases, the proportions allocated to such internal organs as the kidney and liver, and to such tissues as the serum, increase while there is a slight fall in the proportion assigned to the carcass, the part that contains more than 80% of the total protein. But it is not justifiable to generalize from these facts to the statement that the external and peripheral musculature, bone and skin lose and the internal organs gain, for there is no regular or definite change in the alimentary tract, etc., or heart as the protein intake increases. For certain organs at least, what happens may be better understood by considering how the change in conditions may influence not only the economy as a whole, but especially how it may affect the function of each organ in particular. In the case of the kidney we know that every increase in protein consumption above the quantity needed for maintenance and growth requires an increase in the osmotic work of the kidney, and it is reasonable to ascribe

the steady rise from the level on the 11% protein diet in the proportion of the total protein assigned to the kidney to the hypertrophy that follows increased work (Walter and Addis, '39). Although we cannot see clearly how increase in protein consumption may influence the many functions of the liver, it is at least a matter of interest that the hypothesis that serves for the kidney is shown by figure 2 to be inadequate for the liver, since this organ does not increase its share of total protein with luxus protein consumption but declines as the total anabolism diminishes. There are other organs and tissues (e.g., the uterine changes secondary in all probability to an endocrine effect) in which the structural alterations may not be related to change in function. But in every organ and tissue the effect of increasing protein consumption is an individual one so that it is impossible to predict the effect on any part of the body from the change in the protein of the body as a whole.

## SUMMARY

Diets containing 6, 11, 16, 27 and 43% of protein, respectively, that were almost isocaloric and that contained adequate and equal mineral and vitamin concentrations were constructed by substituting casein for cornstarch. These diets were given for 18 days to five groups of rats, twenty rats in each group, each group having at 0 days an identical body weight of 150 gm. On the eighteenth day the protein content of various organs and tissues was determined. A sixth group of rats similar in every respect to the others was killed at 0 days and the change that had occurred in the diet groups was determined by subtracting the quantities found at 0 days from the protein quantities found on the eighteenth day.

The greatest total gain occurred on the diet containing 27% of protein but each organ and tissue had its own mode of reaction to an increasing supply of food protein. Some organs made their maximum gain of protein on the 16%, some on the 27% and others on the 43% protein diet.

Each level of protein intake was associated with its own characteristic pattern of distribution of protein (organ protein per 100 gm. total protein).

## LITERATURE CITED

ADDIS, T., L. J. POO AND W. LEW 1936 The rate of protein formation in the organs and tissues of the body. J. Biol. Chem., vol. 116, pp. 343–352.

ADDIS, T., L. J. POO, W. LEW AND D. W. YUEN 1936 Gravimetric methods for the determination of total body and organ protein. J. Biol. Chem., vol. 113, pp. 497–504.

FORBES, E. B., LEROY VORIS, J. W. BRATZLER AND WALTER WAINIO 1938 The utilization of energy producing nutriment and protein as affected by the plane of protein intake. J. Nutrition, vol. 15, pp. 285–307.

HAMILTON, T. S. 1939 The growth, activity and composition of rats fed diets balanced and unbalanced with respect to protein. J. Nutrition, vol. 17, pp. 565–582.

POO, L. J., W. LEW AND T. ADDIS 1939 Protein anabolism of organs and tissues during pregnancy and lactation. J. Biol. Chem., vol. 128, pp. 69–77.

WALTER, F., AND T. ADDIS 1939 Organ work and organ weight. J. Exper. Med., vol. 69, pp. 467–483.

# IRON AND COPPER VERSUS LIVER IN TREATMENT OF HEMORRHAGIC ANEMIA IN DOGS ON MILK DIETS [1]

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The widespread tendency among clinicians to adduce superior though unexplained properties to liver for treatment of hemorrhagic anemias brought the question to us of whether liver would actually prove superior to iron and copper in treatment of dogs made anemic by hemorrhage and maintained on a whole cow's milk diet. Potter, Elvehjem and Hart ('38) had already shown that nutritional anemia induced in growing dogs by milk feeding is curable by iron and copper therapy. They had also demonstrated the need for copper with iron for maximal hematopoiesis following hemorrhage. We have attempted to answer experimentally several questions raised by their work.

The specific deficiency of iron and copper obtained in dogs on a milk diet is far less complicated than the partial deficiencies of protein (Robscheit-Robbins and Whipple, '37), individual amino acids (Whipple and Robscheit-Robbins, '37), riboflavin (György, Robscheit-Robbins and Whipple, '38), iron (Hahn and Whipple, '36), and copper (Elden, Sperry, Robscheit-Robbins and Whipple, '28; and Sturgis and Farrar, '35), which exert a deterrent effect to normal hemoglobin building in the salmon-bread diet used by Whipple and his co-workers in their well-known anemia experiments with dogs.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

In their work liver supplies all of the individual deficiencies of the salmon-bread diet and produces maximal hemoglobin building, whereas compensation of any individual deficiency, such as riboflavin, for instance, results in only a partial effect. In our work we have felt confident that raw milk supplied all essentials in at least fair amount, except known mineral elements, and that the addition of liver would not enhance its capacity for supporting hemoglobin production appreciably above that obtained by adding the deficient mineral elements. The following experiment was conducted to test this theory.

## EXPERIMENTAL

Six mature litter mate collies were made anemic by phlebotomy. The dogs had been on an exclusive milk diet since birth and had overcome one nutritional anemia, or a nutritional anemia followed by a single hemorrhagic anemia, through iron and copper therapy. The amount of blood drawn in order to lower the hemoglobin level to 6–7 gm. per 100 cc. of blood ranged from 400 to 1000 cc. and was proportional roughly to the size of the dog. Bleedings were made about twice weekly for 5 weeks when the dogs were considered to be in a stable state of anemia. This proved to be the case, since during the subsequent 4-week period when no therapy was given, increases in hemoglobin level were uniformly small and the calculated hemoglobin production was negligible (table 1). Blood volume was assumed to be 8% of the body weight in calculating total hemoglobin.

Dogs 1 and 7 were fed 100 gm. daily of whole dried liver which was sufficient to supply 3 mg. of copper and 100 mg. of iron by analysis. They showed the expected response, arriving at normal levels in 4 weeks. The calculated hemoglobin production was optimal by all standards. Dog 5 which was fed adequate iron (30 mg.) and copper (3 mg.) daily showed equally rapid regeneration and the calculated hemoglobin production per kilogram of body weight was approximately equivalent to that obtained in the case of liver therapy; in three instances, namely dogs 3, 4, and 6, where iron alone was

fed the increases in hemoglobin were slight and the calculated hemoglobin production only about one-third that obtained with liver or with iron and copper. Dog 4, after receiving iron alone for 4 weeks, was given copper in addition to iron. The rate of hemoglobin building trebled in the succeeding 4-week period.

The per cent of iron utilized for hemoglobin formation was calculated from the iron ingested and the iron which appeared

TABLE 1

*Hemoglobin production in dogs after hemorrhage*

| DOG | WEIGHT IN KILOGRAMS | Hb IN GM./100 CC. | CALCULATED TOTAL Hg. IN DOG | DAILY SUPPLEMENT TO MILK DURING 4-WEEK PERIOD | TOTAL Hb PRODUCED IN 4 WEEKS | PER CENT Fe USED IN Hb |
|---|---|---|---|---|---|---|
| | At end of 4-week period | | | | | |
| 1 | 12.3 | 7.6 | 75 | | 11 | |
| | 14.0 | 15.4 | 172 | 100 gm. whole dry liver* | 97 | |
| 7 | 8.9 | 6.7 | 48 | | 5 | |
| | 10.2 | 15.0 | 122 | 100 gm. whole dry liver | 74 | |
| 4 | 14.8 | 7.1 | 84 | 30 mg. Fe | 40 | |
| | 16.5 | 15.6 | 206 | 30 mg. Fe + 3 mg. Cu | 122 | 16 |
| 5 | 10.9 | 6.8 | 59 | | 1 | 49 |
| | 11.6 | 14.3 | 133 | 30 mg. Fe + 3 mg. Cu | 74 | 30 |
| 3 | 12.7 | 6.9 | 70 | | 1 | |
| | 13.4 | 9.8 | 105 | 30 mg. Fe | 35 | 14 |
| 6 | 9.7 | 6.8 | 53 | | 5 | |
| | 11.1 | 8.8 | 78 | 30 mg. Fe | 25 | 10 |

* Vacuum-dried hog liver prepared by Wilson and Company was used. $MnCl_2$ was supplied equal to 1 mg. Mn per dog per day during the experiment.

as new hemoglobin over the 4 weeks of therapy (1 mg. Fe ≎ 0.294 gm. Hb). In the cases where iron alone was fed, utilization to build hemoglobin was not above 16% while in the two instances where copper supplemented iron the per cent utilization was two and three times as great. No correction was made for the small amount of iron supplied by the milk.

Further work has shown that iron utilization is practically nil when copper stores in the animal are reduced to a sufficiently low level. Also the amounts of iron fed in this experiment are considerably above the minimal levels which were

later found to promote rapid hematopoiesis. Both of these factors in this experiment actually operate to minimize the importance of copper.

## DISCUSSION

The difference between our technique and that used by Whipple and co-workers should be borne in mind in evaluating these results. In our work the effect of hemorrhage is superimposed on the effect of dietary conditions which make for an iron and copper deficiency anemia only. The diet appears to supply other essentials in good amount. In the Whipple technique a state of anemia is produced prior to therapy and maintained during therapy by continued bleeding, thus imposing continued demands on the diet for blood-building supplies. Both techniques in certain ways simulate the condition of the commonly occurring hypochromic anemias of infancy, blood loss and pregnancy, and chlorosis. The abnormally great demand placed on the diet for all blood building materials by the Whipple technique has been best met by liver therapy. This has been true because the diet used was deficient in several factors and because liver is notoriously rich in all of the many factors which have been in some way related to the hematopoietic function. The part played by each individual factor contained in liver, such as iron, copper, vitamin $B_6$, and riboflavin must be quite separate and distinct in the complex chain of reactions leading to the in vivo synthesis of hemoglobin. Study of the role of the factors thus far known to play a definite part in hematopoiesis would seem to be best approached by use of diets deficient in only one or two such factors. Milk is admirably suited as a diet deficient in iron and copper, and in cases where iron and copper are supplied it may prove equal to liver in supplying organic factors.

Iron utilization in dogs on a whole milk diet is very complete and under certain conditions may approach 100%. Although Whipple's dogs obtain about 20 mg. of iron per day, calculated utilization of this iron to build hemoglobin is quite low. This suggests that iron utilization is dependent not so

much on the amounts of iron fed, but on the completeness of the diet with regard to other factors influential in the metabolism of iron. These points will be discussed more fully in a subsequent publication.

Presumptive evidence that a deficiency of cobalt in dogs on a whole milk diet may operate at times to retard normal hematopoiesis even in presence of iron and copper has been obtained in this laboratory. In any case the requirement for cobalt must be very small, considerably less than 0.1 mg. per day. In the experiments cited no evidence of a cobalt deficiency was noted.

## SUMMARY

These data clearly support the essentiality of copper with iron for hemoglobin production in dogs and present evidence that whole milk with iron and copper can successfully meet the great increase in demand for blood-forming elements occasioned by severe blood loss.

On a whole milk diet regeneration of hemoglobin was no more rapid with liver therapy than with the iron-copper treatment.

## LITERATURE CITED

ELDEN, C. A., WARREN M. SPERRY, F. S. ROBSCHEIT-ROBBINS AND G. H. WHIPPLE 1928 Blood regeneration in severe anemia. XIII. Influence of certain copper salts upon hemoglobin output. J. Biol. Chem., vol. 79, pp. 577–586.

GYÖRGY, P., F. S. ROBSCHEIT-ROBBINS AND G. H. WHIPPLE 1938 Lactoflavin (Riboflavin) increases hemoglobin production in the normal dog. Am. J. Physiol., vol. 122, pp. 154–159.

HAHN, P. F., AND G. H. WHIPPLE 1936 Iron metabolism. Its adsorption, storage and utilization in experimental anemia. Am. J. Med. Sc., vol. 191, pp. 24–42.

POTTER, V. R., C. A. ELVEHJEM AND E. B. HART 1938 Anemia studies in dogs. J. Biol. Chem., vol. 126, pp. 155–173.

ROBSCHEIT-ROBBINS, F. S., AND G. H. WHIPPLE 1937 Globin utilization by the anemic dog to form new hemoglobin. J. Exp. Med., vol. 66, pp. 565–578.

STURGIS, C. C., AND G. E. FARRAR 1935 Hemoglobin regeneration in the chronic hemorrhagic anemia of dogs (Whipple). I. The effect of iron and protein feeding. J. Exp. Med., vol. 62, pp. 457–465.

WHIPPLE, G. H., AND F. S. ROBSCHEIT-ROBBINS 1937 Amino acids (natural and synthetic) as influencing hemoglobin production in anemia. Proc. Soc. Exp. Biol. and Med., vol. 36, pp. 629–632.